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Studies on Tumor Antigens [1, 2]

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Despite the fact that a great number of investigations have been devoted to tumor immunology (*1,2*), the fundamental problems in this area are far from being solved. One of the most important questions in the field is whether or not malignant tumors possess certain antigens that are not present in corresponding normal tissues. Specific antigens have been found in tumors caused by viruses; attempts to find them in tumors not transmitted by filtrates have yielded positive results in only a few cases (*3–6*).

The present paper deals with some aspects of the work on tumor antigens carried out in this laboratory. The starting point of the experiments to be described was the supposition that specific antigens are present in tumor tissue in low concentrations.

Accordingly, we tried to separate tumor tissue into various components in the hope that specific tumor antigens would be concentrated in one of the fractions. Transplanted tumors were employed that had been induced in rats and mice by chemical carcinogens (in most instances, 9,10-dimethyl-1,2-benzanthracene). The most suggestive results were obtained with protein fractions. Sera from rabbits immunized with tumor tissue showed the complement-fixation reaction with the protein fraction from the same tumor, while sera from rabbits immunized with corresponding normal tissue did not react or reacted only at considerably lower titers with the same tumor protein fraction (*7–9*). Similar results were observed in precipitation experiments (*10*).

These experiments indicated that the protein fractions of tumors and of normal tissues are different in their antigenic composition. However, the results were not consistent and probably depended upon variations in the titer of the sera, the purity of the fraction, etc.

Some time later, a method was developed which made possible the detection of antigens in tumor tissues that were not present in normal tissue. The principle of the method was as follows: Guinea pigs were sensitized with a subcutaneous injection of the tumor protein fraction. After 25 to 30 days, the animals were injected intravenously with a similar protein

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[2] Proof of this article was not examined by the author.

fraction from corresponding normal tissue and were desensitized to this fraction. Two hours later the guinea pigs were injected intravenously with the same tumor fraction that had been used for sensitization (challenge dose). Since the animals had been completely desensitized to the normal fraction, their reaction to the challenge dose demonstrated sensitivity to the tumor fraction. Therefore, the antigenic composition of the tissues under consideration was different.

When this fact was clearly established, a series of experiments was carried out to try to define the nature of the tumor antigens. We were particularly interested in clarifying the question of whether the tumors caused by viruses have some antigen that is not viral in origin and is not present in the tissues of the normal organism. These experiments were done with Shope papilloma and carcinoma, from which the virus was removed by adsorption on rabbit erythrocytes. Extracts of these tumors were compared with each other and with similar extracts from normal rabbit skin, using anaphylaxis following desensitization as an indicator. All these experiments showed that, in addition to viral antigen, the Shope tumor contains tissue antigen that is different from that of normal skin.

Materials and Methods

Preparation of tissue extracts.—Tumors from both animals and human beings were used for the present investigations. The tumor tissues were taken during surgical procedures. If the tumor material was infected (*e.g.*, cancers of the digestive tract) it was washed thoroughly in sterile saline solution, and the tissue for experimentation was taken from the deeper portions. For studies on human leukemic tissue, the material used was spleen obtained at autopsy.

Fresh tumor tissue was freed of necrotic portions, weighed, and then homogenized in a Waring-type blender in a small amount of saline solution until a fine suspension was obtained. The suspension was diluted with physiologic saline (1:3) and the *p*H was adjusted to 8.0 with NaOH solution. The material was then left at 4 to 6° C. for 2 hours with continuous shaking, or until the next day without shaking. The following day, the *p*H of the suspension was checked again and alkaline solution was added, if necessary, to adjust the *p*H to 8.0. The material was then centrifuged for 40 minutes at 4000 r.p.m. The sediment was discarded and acetic acid was added to the supernatant fluid to make the *p*H 6.0. The resulting precipitate was removed by centrifugation and discarded, and the *p*H of the supernatant was adjusted to 4.5 with acetic acid. The precipitate which now appeared was collected by centrifugation, washed in distilled water, adjusted to *p*H 4.5, and then dissolved at *p*H 7.2 by means of distilled water to which a 1 percent solution of NaOH had been added. A milliliter of the protein solution thus obtained was the equivalent of a gram or slightly less of whole tissue.

The entire procedure was carried out under sterile conditions. The material was preserved with merthiolate (1:10,000) or formol (1:500) solution and stored at 4 to 6° C. for not more than 2 months. For sensiti-

zation, only freshly prepared material was used. The nitrogen content and protein concentration were determined by the micro-Kjeldahl method. In a majority of the specimens tested, the amount of protein ranged from 6 to 12 mg. per ml. The materials from normal animals or from normal human beings (those who had died of causes other than cancer) were prepared in the same manner.

The method briefly outlined above was also used by Belozersky (*11*) for extraction of nucleoproteins. The nitrogen-phosphorus ratios in these preparations show that they are not pure nucleoproteins.

Preparation of reactive guinea pigs.—The protein extracted from the tumors by the method described was used to sensitize the guinea pigs. In each experiment 3 to 5 animals were used, each weighing 300 to 350 gm. The extract was administered subcutaneously in a dose ranging from 4 to 10 mg. of protein per animal. The preparations from some tumor tissue (*e.g.*, Shope papilloma) had a very high sensitizing activity, and the dose was accordingly decreased to 1 to 2 mg. of protein.

After 25 to 30 days the guinea pigs were desensitized by intravenous injection of the preparation from a corresponding normal (control) tissue. For example, if sensitization was carried out with material from human hepatoma, the guinea pigs were desensitized with material prepared from normal liver tissue of a person who had died of other causes. The injections were made into the femoral vein through an incision in the skin overlying the vein near the distal end of the hind leg.

Desensitization was begun with intravenous injection of an amount of extract from normal tissue equal to one half or one quarter of the sensitizing dose. The experimental animals usually responded by moderate or slight anaphylactic shock. Larger doses can be used for desensitization, but the shock reaction induced in these instances is so strong that it interferes with recovery. Therefore, it is more convenient to desensitize with a series of small doses.

After the animals recovered completely from shock and their temperature returned to normal (usually within 2 hours after the first desensitizing injection) they were tested for completeness of desensitization with a second injection of normal-tissue protein at the same or a somewhat larger dose. If they did not react they were considered completely desensitized. However, if the second injection was followed by anaphylactic phenomena, a third injection, of the same or a somewhat larger dose than the second, was administered 2 hours later. It was considered desirable to desensitize the animals to the same dose of protein as that used for sensitization, and usually only 2 injections were necessary to achieve this result. Stronger reactions were observed in those cases where the challenge dose of tumor protein was twice as large as the sensitizing one, and in order to have a proper control at this dose level, it was necessary to desensitize the animals to a dose of material from normal tissue equal to that from the tumor.

Sometimes desensitization against normal-tissue antigens was effected after only 2 injections, in increasing amounts, while in other instances a larger number of injections was necessary. For example, guinea pig 231

(table 1) was sensitized by a preparation from mammary-tumor tissue and desensitized by 2 injections of a preparation from normal mammary-gland tissue, whereas for animals 232 and 233 three such injections (3.0, 6.0, and 9.0 mg.) were required for complete desensitization.

It has already been noted that it is desirable to desensitize the animals with repeated small doses in order to avoid strong anaphylactic reactions, from which the animals do not recover for long periods of time. Therefore, in some experiments the animals received 2, 3, or even 4 injections of desensitizing material. Moreover, in the case of highly sensitive animals, the procedure was continued into the following day, the first intravenous dose on the second day being the same as the last dose of the preceding day. Alternatively, on the first day the animals were given a subcutaneous dose twice as large as the sensitizing one, which was followed the next day by intravenous injections in the manner already described.

After the animal was found to be completely desensitized to the desired dose of normal-tissue protein, it was injected with tumor-tissue extract in a dose which did not exceed (in protein content) the last desensitizing one. The animal went into anaphylactic shock, which indicated that the tumor-tissue preparation contained an antigen not present in corresponding normal tissue.

Each preparation from either tumor or normal tissue was tested for both sterility, by culture on nutrient media, and toxicity. In the latter case, the material was injected into the vein of untreated control animals in an amount equal to the largest experimental dose administered. If toxic phenomena were observed, which happened rarely, the material was discarded, as was material found to be nonsterile.

It should be pointed out that in all experiments with human tumors donors of normal and tumor tissue were of the same blood group. Human carcinomas of the following sites were studied: liver, stomach and esophagus, mammary gland, parotid gland, pancreas, ovary, uterus, lung, and bladder (including papilloma), and several sarcomas; and in addition, spleen tissue from patients who had died of different forms of leukemia. All tissues were examined histologically.

In addition to the primary tumors listed, tumor tissues from metastases in other organs were also investigated. For metastatic tumor tissue, desensitization was carried out with extracts from normal tissues corresponding in type with the tissue in which the tumor had originated and the tissue to which it had metastasized. For example, guinea pigs sensitized to protein from the liver metastasis of a primary gastric carcinoma were desensitized by materials from both normal mucous membrane of the stomach and normal liver.

Preparation of virus-free Shope papilloma extracts.—The Shope papilloma, a viral tumor, was received in 1939 from the Rockefeller Institute in New York and adapted to domesticated rabbits at the Medical-Biological Station in Sukhumi. The virus is inoculated into domesticated rabbits as a fine suspension of papilloma tissue and is preserved well in

glycerine. However, in the domesticated animals the activity of the virus is decreased considerably as compared to that of the strains maintained in wild rabbits. Positive takes are obtained with tissue suspension at dilutions up to 1:100.

To free the papilloma extract from virus, the papilloma tissue was first ground in a mortar with quartz sand and then diluted with 4 times the amount of saline solution. The suspension was thoroughly mixed and the *p*H adjusted to 7.4; it was shaken repeatedly for 2 hours at a temperature of 6 to 7° C. The material was then centrifuged for 20 minutes at 4000 r.p.m. The resulting supernatant fluid contained Shope virus; it was then treated as follows.

Zilber *et al.* in 1953 (*12*) noted that Shope virus can be adsorbed on the erythrocytes of different animal species. Our method for freeing the papilloma extract of virus was based on this observation. Washed rabbit erythrocytes were introduced into the papilloma extract, which had been adjusted to *p*H 6.6, in a ratio of 1:4, and the suspension was allowed to stand for 30 minutes at 37° C. The erythrocytes were then removed by centrifugation. This procedure was repeated 4 times in all. As a rule, after the fourth time, the supernatant contained no demonstrable virus, since upon inoculation into rabbit skin it did not yield papillomas. At the same time papillomas developed when the erythrocytes, which were washed thoroughly after contact with the papilloma extract, were applied to scarified rabbit skin.

Some hemolysis occurred in the papilloma extract-erythrocyte mixture, and the presence of hemoglobin was undesirable for immunologic analysis and quantitative estimation of the protein. Therefore, in the method developed later, intact red blood cells were replaced by their stroma. Washed red blood cells were mixed with distilled water (1:4), thoroughly shaken, and centrifuged at 12,000 r.p.m. The sediment, consisting of erythrocyte stroma, was washed with 0.15 percent saline, isotonic to the hemolyzing solution, and was then used for adsorption. The amount of stroma used was equated with the volume of erythrocytes which had been used previously.

Experiments with papilloma extracts.—The virus-free extracts were used to sensitize the guinea pigs according to the method described earlier. The doses of antigens were estimated by and controlled according to the protein content.

Desensitization of the guinea pigs was carried out with extracts from normal rabbit skin prepared in the same manner as the papilloma extracts, using erythrocyte stroma for adsorption.

It is necessary to keep in mind that even with the quadruple-adsorption treatment of papilloma extracts virus could still be present in amounts insufficient to produce papillomas in rabbits. Furthermore, a special investigation made in this laboratory by Nartsissov *et al.* (*13*) revealed that the papilloma strain with which we were dealing contains soluble virus antigen. Kidd (*14*) did not find such an antigen, but he used another virus strain that was not adapted to domesticated rabbits.

In accordance with the above considerations, the guinea pigs were desensitized not only to the extracts from normal tissue but also to virus adsorbed on erythrocytes or on the stroma. After complete desensitization to both tissue and virus, the guinea pigs were challenged with the virus-free extract. In addition to the comparative study of antigens in papilloma and normal skin, we carried out a series of experiments in which the same methods were applied to a comparison of the antigenic content in papilloma and in carcinoma of the skin.

Results

To illustrate the results obtained let us consider some of the experiments recorded in table 1.

The table shows that the guinea pigs sensitized by tumor-tissue extracts and completely desensitized to extracts from normal tissue responded to a challenge injection of tumor extract. It follows that the tumor tissue possesses antigens lacking in the normal-tissue extracts. The experiments under consideration were carried out by myself (*15,16*) and repeated by a number of my assistants (*17–21*), and the results observed were essentially the same—animals sensitized by preparations from tumor tissue and completely desensitized to corresponding preparations from normal tissue always showed anaphylactic reactions following the challenge injection of the tumor-tissue extracts. Negative results were observed only in a few experiments in which the tumor used had been treated previously with X rays.

The reverse type of experiment was also carried out, in which guinea pigs were sensitized to preparations of normal tissue and then desensitized with preparations of tumor tissue. Challenge of such animals with preparations from normal tissue did not produce anaphylactic phenomena.

In collaboration with Parnes (*22,23*) analogous investigations were carried out with spleen extracts from human beings who had died of leukemia.

The guinea pigs were sensitized by subcutaneous administration of material from the spleens of patients who had died of acute or chronic myelosis, myeloblastosis, erythroblastosis, and lymphadenosis. The extracts were prepared according to the method already described. Desensitization was carried out by intravenous administration of preparations, extracted in the same way, from the spleens of persons who had died of other causes. In each experiment of this series donors with and without neoplastic disease were of the same blood group. The challenge material in each experiment was the same extract as the one used for sensitization.

Some of the data obtained from these experiments are given in table 2. As the table indicates, guinea pigs that were sensitized by extracts of spleen from leukemic donors reacted to the sensitizing preparations after they had been desensitized by extracts of spleen from normal donors of the same blood group. Therefore, there is good reason to conclude that

TABLE 1.—*Results of desensitization, measured by anaphylaxis, and administration of challenge dose in guinea pigs**

Guinea pig no.	Sensitization		Desensitization			Test for completeness of desensitization			Challenge injection		
	Preparation	Dose (*mg. protein*)	Preparation	Dose (*mg. protein*)	Reaction	Preparation	Dose (*mg. protein*)	Reaction	Preparation	Dose (*mg. protein*)	Reaction
269	Cancer of the stomach	4.0	Normal stomach mucous membrane	2.25	++	Normal stomach mucous membrane	4.5	−	Cancer of the stomach	4.0	++
270		4.0		2.25	+		4.5	−		4.0	+
271		4.0		2.25	++		4.5	−		4.0	+++
260†		—		—			—			4.0	−
231	Mammary cancer	8.5	Normal mammary gland	4.4	++	Normal mammary gland	8.8	−	Mammary cancer	8.5	++
232		8.5		3.0	+		6.0	+			
							9.0	−		8.5	+++
233		8.5		3.0	+		6.0	+			
							9.0	−		8.5	++
220†		—		—			—			8.5	−
284	Cancer of the liver	9.2	Normal liver	4.2	+	Normal liver	10.5	++	Cancer of the liver		
							10.5	−		10.5	++
285		9.2		4.2	++		6.3	−			
							9.4	−		9.2	++
286		9.2		4.2	+		8.4	+			
							12.6	−		12.6	+++
280†		—		—			—			10.5	−
290	Cancer of the ovary	3.0	Normal ovary	1.6	+	Normal ovary	3.2	−	Cancer of the ovary	3.0	++
291		3.0		1.6	+		3.2	−		3.0	++
292		3.0		1.6	++		3.2	−		3.0	+
289†		—		—			—			3.0	−
294	Cancer of the lung	3.2	Normal lung	1.7	+++	Normal lung	2.55	+	Cancer of the lung		
							4.25	−		4.25	++
295		3.2		0.85	+		1.7	+			
							3.4	−		3.4	++
296		3.2		0.85	+		2.55	+			
							4.25	−		4.25	−
293†		—		—			—			4.25	−

*Key for symbols used in tables 1, 2, 3, 5, and 6:
−No anaphylactic phenomena observed.
+Fall of temperature, scratching of nose.
++Scratching of nose fairly pronounced, sneezing, coughing.
+++Strongly pronounced symptoms of shock.
++++Lethal shock.
†These animals were used only for testing the toxicity of the material.

TABLE 2.—*Results with spleen-tissue antigens**

Guinea pig no.	Sensitization		Desensitization			Test for completeness of desensitization			Challenge injection		
	Preparation	Dose (*mg. protein*)	Preparation	Dose (*mg. protein*)	Reaction	Preparation	Dose (*mg. protein*)	Reaction	Preparation	Dose (*mg. protein*)	Reaction
64	Spleen from patient with myeloblastosis	12.0	Normal spleen	7.5	++	Normal spleen	12.0	−	Spleen from patient with myeloblastosis	12.0	++++
65		12.0		9.0	++		13.0	−		10.8	++++
66		12.0		10.5	++		13.0	+		12.0	++
67		12.0		10.5	++		12.0	−			
							12.8	+			
							12.0	−		12.0	+++
68		12.0		10.5	++		12.0	−		12.0	++
69†		—		—			—			12.0	−
82	Spleen from patient with erythroblastosis	7.8	Normal spleen	3.8	+	Normal spleen	7.6	+	Spleen from patient with erythro-blastosis	11.7	+++
							11.4	−			
83		7.8		3.8	++		7.6	+			
							11.4	−		11.7	+++
84		7.8		3.8	+++		7.6	−			
							15.2	−		11.7	++
85		7.8		5.7	++		11.4	+			
							15.2	−		11.7	+
86		7.8		5.7	++		11.4	+			
							15.2	−		15.6	++++
87†		—		—			—			15.6	−

*For symbols used see key in *footnote, table 1.
†These animals were used only for testing the toxicity of the material.

there are antigens present in leukemic spleens which are missing in normal spleens.

Many similar experiments were performed with different animal tumors, including the Ehrlich mouse carcinoma, transplantable mouse mammary adenocarcinoma induced by methylcholanthrene, transplantable mouse sarcomas induced by 9,10-dimethyl-1,2-benzanthracene, Crocker mouse sarcoma 180, a mouse hepatoma induced by 4-*o*-tolylazo-*o*-toluidine (*o*-aminoazotoluene), 3 transplantable rat sarcomas induced by 9,10-dimethyl-1,2-benzanthracene, a spontaneous rat mammary adenocarcinoma, Brown-Pearce rabbit carcinoma, Shope rabbit carcinoma, and a monkey sarcoma, induced with a carcinogen in Petrov's laboratory (*24*). A number of animal tumors of viral etiology, including Rous sarcoma, Shope papilloma, and a strain A mouse mammary carcinoma were also studied.

In all the experiments of this series (*25–28*) the results were essentially similar to those described previously. It is important to note, however, that in studies with sarcomas it was not possible to maintain the same rigorous standards of paired selection of neoplastic and normal control material as in the studies with human carcinoma, mainly because of the limited availability of normal connective tissue for desensitization in the organs from which the sarcomas were taken. As we have already stated, carcinomatous tissue was always compared with its normal counterpart, *e.g.*, gastric adenocarcinoma was compared with mucous membrane of normal stomach, hepatoma with normal liver tissue, etc. Furthermore, donors of both tumor and normal tissue were always of the same blood group.

In the experiments with human sarcoma, animals sensitized with preparations from soft-tissue sarcomas were desensitized with preparations from normal organs rich in connective tissue, such as lung and spleen, and the desensitizing doses were twice as large as the sensitizing ones. The challenge dose usually was equal to the sensitizing one. The same modifications apply to the work with animal sarcomas.

For comparison of the antigenic content of animal epithelial tumors and normal epithelium the tissues chosen were as nearly identical as possible. For example, in experiments with mouse mammary carcinomas, the animals were desensitized with preparations from normal mammary glands, which in some instances had been stimulated by an estrogen. Similarly, in experiments with the Brown-Pearce carcinoma, the preparations for desensitization were extracted from the parietal layer of the rabbit peritoneum, and in the experiments with Shope papilloma the desensitizing material was extracted from the epidermal layer of skin, scraped off with a glass plate. In a number of experiments with Shope papilloma stimulation of proliferation of normal rabbit skin to hyperplasia made possible the collection of a sufficient amount of control material. Differences of blood group were not considered in the investigations with animals.

All the experiments with tumors outlined above were carried out in

TABLE 3.—*Experiments with C3HA mouse hepatoma**

Guinea pig no.	Sensitization		Desensitization			Test for completeness of desensitization			Challenge injection		
	Preparation	Dose (*mg. protein*)	Preparation	Dose (*mg. protein*)	Reaction	Preparation	Dose (*mg. protein*)	Reaction	Preparation	Dose (*mg. protein*)	Reaction
511	Hepatoma	3.0	Normal liver	1.4	++	Normal liver	3.5	−		3.0	+
543		4.0		2.0	+		6.0	−		4.8	++
545		4.0		2.0	+		6.0	−	Hepatoma	6.0	++
546†		—		—			—			6.0	−
216	Hepatoma	3.6	Normal liver	1.4	++	Normal liver	3.5	−		3.6	++
217		3.6		1.4	++		4.2	−	Hepatoma	4.5	+++
218		3.6		1.4	+		4.2	−		3.6	++
219†		—		—			—			4.5	−
111	Hepatoma	6.4	Normal liver	3.0	+++	Normal liver	6.3	−	Hepatoma	6.4	+
112		6.4		2.25	++		6.3	+		6.4	++
							6.3	−			
113		6.4		2.25	+		4.5	+			
							6.3	−		6.4	++
114†		—		—			—			6.4	−

*For symbols used see key in *footnote, table 1.
†These animals were used only for testing the toxicity of the material.

noninbred animals. In 1952, Hauschka (*2*) published data indicating that some antigenic peculiarities of tumor tissue are dependent upon genotypic differences among the animals in which they arose. These findings prompted a series of experiments, in collaboration with Levina (*28*), on genotypically homogeneous animals. The sensitization-desensitization procedures were carried out with mouse hepatoma which had arisen in one of the C3HA stock mice fed 4-*o*-tolylazo-*o*-toluidine and had been transplanted into mice of the same strain. Table 3 illustrates the results obtained.

The table shows that the animals sensitized by material from the hepatoma and desensitized by material from normal mouse liver responded by shock to the challenge injection of hepatoma preparation. The results indicate that hepatoma tissue of C3HA mice also contains antigen lacking in normal liver in the same strain of animals.

From the data obtained the question of the nature of the antigens under consideration has naturally arisen. It might be suggested that they represent normal tissue proteins transformed during the process of malignization. On the other hand, the possibility is not excluded that the tumor-specific antigens are the masked viruses themselves. Therefore, it was important to study this problem first of all with respect to the tumors whose viral nature is now beyond doubt. The viruses can be removed from these tumor extracts by several methods, and comparative investigations of the antigenic content of virus-free tumor extracts and extracts from the corresponding normal tissue might yield information about the possible presence of additional antigens of tissue origin. Experiments of this type with Shope papilloma were carried out (*29*) and the data are presented in table 4.

The table shows that the erythrocyte stroma are able to adsorb the papilloma virus.

The ability of rabbit erythrocytes to adsorb the virus was also shown by Nartsissov *et al.* (*30*) in complement-fixation experiments. In biologic determinations, the extract from papilloma tissue, after fourfold contact

TABLE 4.—*Experiments with adsorption of Shope papilloma virus on rabbit erythrocytes*

Expt. no.	Initial material	Material tested in biological experiments	Development of papilloma (*days after inoculation*)
47	Extract from papilloma tissue	Initial extract.	38
		Stroma after adsorption.	46
		Supernatant fluid after 4th adsorption.	None
51	Same	Initial extract.	39
		Stroma after adsorption.	48
		Supernatant fluid after 4th adsorption.	75*
62	Same	Initial extract.	30
		Stroma after adsorption.	47
		Supernatant fluid after 4th adsorption.	None

*By the 82nd day the papilloma had regressed.

TABLE 5.—*Comparison of antigenic peculiarities of*

Expt. no.	Sensitization		Desensitization					
			Normal antigen					
			First desensitizing injection†			Test for completeness of desensitization		
	Antigen	Dose (*mg. protein*)	Antigen	Dose (*mg. protein*)	Reaction	Antigen	Dose (*mg. protein*)	Reaction
7	Papilloma extract after adsorption on erythrocyte stroma	2.0	Extract from normal skin after adsorption with erythrocyte stroma	0.5	+++	Extract from normal skin after adsorption on erythrocyte stroma	2.0	+
8		"		"	++		"	−
9		"		"	++		"	−
10		"		0.7	+++		"	−
11		"		0.5	++		"	−
12		"		"	++		"	−
13		"		"	+++		"	−
14		"		"	+++		"	−
15		"		"	+++		"	−
16		"		"	+++		"	−
17		"		"	++		"	−
18		2.5		1.0	+++		2.5	−
19		"		"	++		"	−
20		"		"	+++		"	−
21		"		"	++		"	−
22		"		"	+++		"	−

*For symbols used see key in * footnote, table 1.
†Subsequent desensitizing injections are not given in the table.

with the rabbit erythrocyte stroma, did not contain virus in the majority of samples tested.

In table 5 are presented the results of experiments in which comparison was made between the antigenic content of papilloma tissue and normal rabbit skin. The data show that the guinea pigs sensitized by the extract from papilloma tissue and desensitized by the extract from normal skin remained sensitive to papilloma virus. This fact points to the presence in the sensitizing papilloma extract of either a small amount of the virus, insufficient for detection in biological experiments, or a soluble viral antigen.

However, when the same guinea pigs were completely desensitized to the viral component by injection of the virus adsorbed on the erythrocyte stroma, they were found to be sensitive only to the extract by which they were sensitized. Although the anaphylactic reactions observed in these experiments were moderate they were, nevertheless, observed consistently, which seems to indicate that papilloma and normal-skin antigens are different in their composition.

Considerably greater differences were found in the antigenic content of papilloma tissue and of cancer which had developed from papilloma. These data are presented in table 6, and show clearly that the tissues of papilloma and carcinoma contain additional antigen, missing in normal rabbit skin, that is distinct from the viral antigen.

Other indirect evidence regarding the papilloma tissue was obtained from the experiments of Postnikova (*31*). In her study, a suspension

*papilloma tissue and normal rabbit skin**

Desensitization—Continued						Challenge injection		
Viral antigen								
First desensitizing injection†			Test for completeness of desensitization					
Antigen	Dose (*ml. stroma*)	Reaction	Antigen	Dose (*ml. stroma*)	Reaction	Antigen	Dose (*mg. protein*)	Reaction
Stroma after adsorption of virus‡	0.5	+	Stroma after adsorption of virus‡	1.5	−	Papilloma extract after adsorption with erythrocyte stroma	2.0	++
	"	+		"	−		"	+
	0.75	+		"	−		"	+
	"	+		"	−		"	+
	"	+		"	−		"	+
	"	++		"	−		"	−
	"	++		"	−		"	+
	"	+		"	−		"	+
	1.0	+		2.0	−		"	+
	"	++		"	−		"	+
	"	++		"	−		3.0	++
	1.5	+		"	−		"	−
	1.0	+		"	−		"	−
	0.5	−		"	−		"	+
	1.0	+		"	−		"	+
	"	+		"	−		"	+

‡One ml. of stroma suspension after adsorption contained the adsorbed virus from 1 ml. of initial virus-containing extract.

of papilloma tissue containing active Shope virus was inoculated into the scarified skin of rabbits bearing papillomas. In a few days some papilloma tissue was autotransplanted into the muscle of the leg. In 12 out of 19 experimental animals the tumor autotransplants grew, while no papillomas developed at the site of inoculation of the viral suspension, which had produced papillomas in a group of control rabbits. These results suggest that immunity to the papilloma virus is produced by antigens different from those involved in immunity to the cells of papilloma tissue.

More extensive investigation of this problem was undertaken in this laboratory by Baydakova and Radzichovskaya (*32*). They immunized Leghorn hens against Rous sarcoma virus, as follows: Rous sarcoma was homogenized in saline solution (1:15) to which 10 percent horse serum was added; the homogenate was then centrifuged and passed through a Berkefeld filter after inactivation with 0.2 percent formol solution. The filtrates were injected in the muscle, in a series of doses of 1, 2, 3, 3, and 3 ml. given at intervals of 3, 3, 3, and 14 days, respectively. After 14 to 22 days had elapsed, the hens were tested for immunity by injection of active Rous virus. Twenty-two out of 57 immunized hens did not develop tumors during 2 months of observation, whereas 35 hens of this series developed sarcomas. In control experiments, administration of the same doses of active virus yielded sarcoma in 32 out of 35 nonimmunized hens and in 5 out of 5 hens injected with filtrate from normal hen muscle.

The hens immune to Rous sarcoma virus were tested for immunity to

TABLE 6.—*Comparative study of extracts from papilloma and cancer tissues**

Sensitization		Desensitization				Test for completeness of desensitization			Challenge injection			
Preparation	Dose (*mg. protein*)	Preparation	Dose (*mg. protein*)	No. guinea pigs	Reaction	Preparation	Dose (*mg. protein*)	Reaction	Preparation	Dose (*mg. protein*)	No. guinea pigs	Reaction
Cancer	3.0	Papilloma	1.2	4	+	Papilloma	3.0	−	Cancer	3.0	4	+
				2	++					3.0	2	++
Cancer	2.0	Papilloma	0.3	4	++	Papilloma	2.0	−	Cancer	2.0	3	++
				2	+		3.0	−		3.0	2	+++
										3.0	1	++++
Cancer	1.5	Papilloma	0.25	6	+	Papilloma	1.5	−	Cancer	1.5	4	+
				2	++		2.5	−		2.5	3	++
										2.5	1	+
Papilloma	3.0	Cancer	1.5	7	+++	Cancer	3.0	−	Papilloma	3.0	3	+++
				4	++					3.0	4	++
										3.0	4	+

*For symbols used see key in *footnote, table 1.

the tumor cells. For this purpose, 0.2 to 0.5 ml. of a 10 percent suspension of tumor cells was injected in 16 of 22 immune animals. All developed sarcomas within the same period of time as the controls inoculated with active virus.

Therefore, immunity against the virus is caused by different antigens than is immunity to tumor cells. Thus, cells of viral tumors contain, in addition to the viral antigen, still other antigens that are missing in the tissues of normal animals.

Discussion

The experiments briefly outlined indicate that, with the help of the anaphylactic reaction following desensitization, it is possible to demonstrate the presence in human and animal tumors of antigens that are missing in corresponding normal tissue. Although our protein fractions represented a mixture of many antigens, the amounts of which may well be different in tumor and normal tissues, it would be difficult to accept the suggestion that the observed differences of antigenic content in tumor and normal tissues are merely quantitative. If the latter is true, then normal protein must be present in greater amounts in tumor tissue than in normal tissue. This seems highly improbable. It appears more likely that tumor tissue contains antigens lacking in normal tissue. The success of the experiments in question is clearly due to a highly sensitive reaction that permits the detection of insignificant amounts of a particular protein in a mixture containing large amounts of other proteins.

The fact that we did not use the total protein extracts of tissue but fractions that precipitated at *p*H 4.5 was undoubtedly significant also. The method of obtaining fractions from tumor and normal tissues for sensitization and desensitization was used by Belozersky (*11*) for the extraction of nucleoproteins. We applied this method to our studies because we felt that if nontransmissible tumors contained masked viruses, they would be concentrated in the nucleoprotein fraction. That tumor-producing viruses are actually precipitated in that fraction was shown by Radzichovskaya (*27*) and Medvedev (*33*). Using the same method, these authors extracted the nucleoprotein fractions from Rous sarcoma and a mouse mammary carcinoma and found that they contained the corresponding viruses in a high concentration.

Since the high sensitivity of the anaphylactic reaction readily demonstrated organ and tissue specificity (*34*), it was necessary to compare the antigenic components of tumors only with components of the corresponding normal tissue from which the tumors had originated. It has already been mentioned that this requirement could not be fulfilled in all the experiments, especially those involving sarcomas. However, the requirement was fully met in many other cases, especially in the experiments with human hepatomas and with tumors of inbred mice.

In 1950 the Academy of Medical Sciences appointed a special commission under the chairmanship of Professor L. Shabad, Corresponding Member of the Academy, to verify these data, and this commission completely confirmed the results (*35*).

After our investigations had been published in Russian, studies were published by Weiler (*36,37*), showing the presence in rat hepatoma of an antigen lacking in the liver of normal rats. Fink, Snell, and Kelton (*38*), using the anaphylactic reaction, also found tumor antibodies in experiments with inbred mice. And with the same tool, Makari (*39*) detected tumor antigens in the plasma of cancer patients.

Investigation of the antigenic content of some viral tumors showed that 2 kinds of antigens are present in the tumor cells that are missing in normal tissues—the viral and the tissue antigens. These different antigens are responsible for different immunizing mechanisms, one being against the virus itself and the other against the tumor cells. This suggestion was first made by Rous as early as 1913 (*40*).

It has been shown repeatedly [*e.g.*, Hauschka (*2*)] that some immunologic characteristics of tumor cells are due to genotypic differences between the transplanted tumor cells and the cells of the host. But, in addition to these characteristics whose existence is beyond doubt, the existence of specific tumor antigens connected with malignization is very probable. The nature of these antigens requires further research. The most important aspect is the study of antigens from human tumors. Preliminary data obtained in this area show that some specific antigens from human tumors can be adsorbed on erythrocytes, while the antigens from transplanted animal tumors induced by carcinogens do not possess this property.

Conclusion

1) The anaphylactic reaction to tumor fractions evoked in animals desensitized by normal homologous extracts makes possible the detection of antigens in tumors of animals and of human beings. These antigens are missing in corresponding normal tissues.

2) The antigens in question can also be detected in tumors induced in inbred mice by carcinogenic substances (hepatoma induced in C3HA mice by 4-*o*-tolylazo-*o*-toluidine).

3) In Shope papilloma, as well as in carcinoma that develops from this papilloma, there is present, in addition to the viral antigen, tissue antigen missing in normal rabbit skin.

4) In the experiments with Shope papilloma and Rous sarcoma the observed differences in the immunizing mechanisms against tumor cells and viruses indicate that both tumors possess specific tissue antigens, in addition to viral antigens.

5) In contrast to tissue antigens, the viral antigen from Shope papilloma can be adsorbed on erythrocytes of rabbits and other animals.

6) The data presented reveal the complex nature of tumor antigens and emphasize the need for further investigation of the problem.

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The Biological Effects of Some Podophyllin Compounds and Their Dependence on Chemical Structure [1]

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Although records of the use of the resin of *Podophyllum peltatum* as a purgative and cholagogue extend back to ancient and medieval times, comparatively few reports have appeared in the literature referring to the true biological activity of podophyllin, a complex of effective substances contained in this resin.[3]

The first extensive study on the biological effect of the resin was published by Podwyssotzki (*2*) in the years 1880–81. He stated that the true active substance, isolated and called podophyllotoxin by him, when treated with alkali isomerized to picropodophyllin, which had no biological activity. Furthermore, Podwyssotzki was able to prepare a podophyllic acid, which also proved to be nontoxic.

Neuberger (*3*) investigated the biological effect of crystalline podophyllotoxin in frogs, rabbits, cats, dogs, pigeons, and chickens. He reported particularly on its pathologic-anatomic effects in these animals. Its toxic action is, to a large extent, comparable with the toxic effect produced by colchicine and some of its derivatives [reviewed by Fühner (*4*)]. Neuberger's observations suggest to us that animals fed meat are far more sensitive to this substance than animals fed plant food only.

Following publication of that paper, no significant reports on the biological effect of this resin were published until 1942, when Kaplan (*5*) presented his observation that the substances isolated from the resin of *Podophyllum peltatum* inhibited the growth of condyloma. King and Sullivan (*6,7*) studied this effect and were the first to draw attention to the analogous effect of podophyllin and colchicine on the dividing cell.

The mitotic and experimental pathologic effects were further investigated with podophyllotoxin, picropodophyllin, and even with crude podophyllin (*1,8–15*). In these investigations it was found that the lethal dose of podophyllotoxin in guinea pigs was lower than 20 mg. per kg., but

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[3] A review on the experimental and clinical studies to date has been presented by Margaret G. Kelly and Jonathan L. Hartwell (*1*).

for picropodophyllotoxin it was higher than 50 mg. per kg. The mitotic effect of both compounds was studied in the cornea with the Nipple test by application of different concentrations of the compounds in the form of a paste and histologic examination of the tissue.

The establishment of the mitotic effect of this crude material attracted the attention of chemists, as well as biologists, so that within a short time a number of other substances were isolated from this plant material and further derivatives prepared from some of them (*1,16–20*). Hartwell and his group succeeded in obtaining new substances that were closely related to podophyllotoxin. These newly isolated substances also isomerized very easily into the so-called B series, which corresponded to picropodophyllin (table 1).

Experiments carried out in white mice inoculated with sarcoma 37 proved (*21*) that the 3 main components, podophyllotoxin, α-peltatin-A, and β-peltatin-A had the same maximum tolerated dose (30–40 μg./gm.) and minimum effective dose (2 μg./gm.). But a report on the lethal dose and the pathologic-anatomic picture produced by administration of the 2 substances was not presented by the authors.

Because the work at our Institute was concerned with certain biological effects of the substances derived from the resin of *Podophyllum peltatum*—podophyllotoxin, α-peltatin-A, β-peltatin-A, 4′-demethylpodophyllotoxin, deoxypodophyllotoxin—we decided to investigate their LD50 and LD100 in white rats, and in addition, to study the pathologic-anatomic picture produced in these animals. Furthermore, we examined their effect on mitosis in regenerating rat liver in order to calculate the toxicity-stathmokinesis index. From this index value, conclusions may be drawn about the suitability of the preparation for therapeutic use against cancer. Finally, we decided to investigate the derivatives of these components as well, in order to establish the degree of dependence of their biological activity on their chemical structure.

Materials and Methods

The substances used in these experiments were isolated by chromatographing resina podophylli, which originates from duck's-foot (*Podophyllum peltatum* L.) (*16*). From the compounds obtained, we were able to prepare the necessary derivatives.[4]

For all experiments male and female Wistar rats, weighing 100 to 250 gm., were used. They were given a single subcutaneous injection of a water-alcohol or propylene glycol solution of the compound. The results were identical in both cases. Experiments on controls were carried out simultaneously with the same amount of solution. All the substances utilized for this work proved to be insoluble in water.

The LD50 for each dose was determined in 30 rats (*22*), according to the method of Trevan, and the LD100, in at least 10 rats for each dose.

[4] For some of the derivatives we are indebted to Dr. J. L. Hartwell, National Cancer Institute, Bethesda, Md., to whom the authors express sincere thanks.

Autopsies were performed and the parenchymatous organs prepared for histologic evaluation in the usual fashion (they were fixed in 10% formol, embedded in paraffin, and the sections stained with hematoxylin and eosin).

The stathmokinetic [5] effect was determined by studying regenerating rat livers (*23*), a method which had previously enabled us to obtain good results in studies of the effect of colchicine derivatives (*24–26*) and made possible comparisons of the mitotic activity of colchicine with podophyllin derivatives. Twenty-seven hours after partial hepatectomy, the substances under investigation were injected subcutaneously; 18 hours later (*i.e.*, 45 hours after surgery), the rats were decapitated and the livers removed and examined histologically. The animals were always hepatectomized at the same time, and the intervals between surgery, injection, and sacrifice were kept constant to avoid discrepancies which might be due to diurnal periodicity in mitotic activity, as pointed out by Jaffe (*27*).

Results and Discussion

Toxicity and Pathologic-Anatomic Findings

Table 1 summarizes the data on *toxicity* of all the substances under investigation. The values obtained indicate that both the LD50 and LD100 are virtually the same for podophyllotoxin and α- and β-peltatin-A. Comparison of the structure of the compounds in relation to their toxicity shows that the substituents of the A, B, and C rings, which have undergone a change in our compounds, do not produce a substantial effect on biological activity, except for the configuration of the lactone ring, which is considered to have the *trans*-configuration in these compounds.

Varied results with respect to toxicity were obtained for acetylated or methylated derivatives of the 3 substances; while the toxicity exhibited by acetylated podophyllotoxin is about 15-fold less than that of podophyllotoxin, with α- and β-peltatin-A, there is a two- or threefold decrease in toxicity.

It is of considerable interest that the methylated peltatins of the A series are nontoxic, as are deoxypodophyllotoxin, which, unlike podophyllotoxin, has no hydroxyl group in position 8, epipodophyllotoxin, which has the hydroxyl group in the *cis*-configuration to the substituent at C_7, and, finally, podophyllotoxon, which has a carbonyl group at C_8.

4′-Demethylpodophyllotoxin and α-peltatin-A exhibit demethylation of the same phenolic group of the C ring, but the former has been found to be less toxic than the latter or than podophyllotoxin.

All these substances also proved to be nontoxic when transferred to the B series, which confirms Podwyssotzki's (*2*) observations and makes them applicable to other podophyllin compounds. In this case, the loss of

[5] As a rule the term "stathmokinesis" is applied to those pathologic forms of mitosis in which, after mitotic arrest of cell division in the metaphase, dispersion of the chromosomes or their fragments is found throughout the cytoplasm, which generally appears enlarged and stains more lightly.

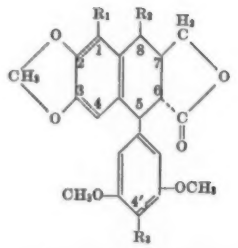


TABLE 1.—*Compounds investigated and values obtained*

Investigated compounds	Configuration of substituents of B ring	Changing substituents of A, B, and C rings			Results				
					Toxicity		Mitotic effect		Toxic-stathm. index*
		R_1	R_2	R_3	LD100 (*mg./kg.*)	LD50 (*mg./kg.*)	Dose (*mg./kg.*)	Stathm. index	
Podophyllotoxin	*trans*(8:7)*trans*(7:6)*cis*(6:5)	H	OH	OCH_3	40	14	15	138	2.7
Podophyllotoxin acetate	"	H	$OOCCH_3$	OCH_3	200	185	100	8	<2
Picropodophyllin	*trans*(8:7)*cis*(7:6)*trans*(6:5)	H	OH	OCH_3		>500	>200	0	0
Picropodophyllin acetate	"	H	$OOCCH_3$	OCH_3		>500	>200	0	0
Epipodophyllotoxin	*cis*(8:7)*trans*(7:6)*cis*(6:5)	H	OH	OCH_3		>200	>200	0	0
Epipicropodophyllin	*cis*(8:7)*cis*(7:6)*trans*(6:5)	H	OH	OCH_3		>200	>200	0	0
4′-Demethylpodophyllotoxin	*trans*(8:7)*trans*(7:6)*cis*(6:5)	H	OH	OH	75	30†	50	18	1.5
Podophyllotoxon	*trans*(7:6)*cis*(6:5)	H	O	OCH_3		>200	>200	0	0
Deoxypodophyllotoxin	"	H	H_2	OCH_3		>200	>200	0	0
β-Peltatin-A	"	OH	H_2	OCH_3	25	12	15	27	1.7
β-Peltatin-A-acetate	"	$OOCCH_3$	H_2	OCH_3	70	25	35	13	2.0
β-Peltatin-B	*cis*(7:6)*trans*(6:5)	OH	H_2	OCH_3		>500	>200	0	0

β-Peltatin-B-acetate	"	$OOCCH_3$	H_2	OCH_3		>500	>200	0	0
α-Peltatin-A	*trans*(7:6)*cis*(6:5)	OH	H_2	OH	30	17	20	6	1. 5
α-Peltatin-A-diacetate	"	$OOCCH_3$	H_2	$OOCCH_3$	100	65	60	42	1. 7
α-Peltatin-B	*cis*(7:6)*trans*(6:5)	OH	H_2	OH		>500	>200	0	0
α-Peltatin-B-diacetate	"	$OOCCH_3$	H_2	$OOCCH_3$		>500	>200	0	0
α, β-Peltatin-A-methyl ether	*trans*(7:6)*cis*(6:5)	OCH_3	H_2	OCH_3		>200	>100	0	0
α, β-Peltatin-B-methyl ether	*cis*(7:6)*trans*(6:5)	OCH_3	H_2	OCH_3		>200	>100	0	0

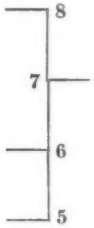
*Calculated by dividing the value for LD100 by that for the mitotically effective dose.
†Because of insufficient quantities only approximate values were obtained.

effectiveness is conditioned by modification of the lactone ring from the *trans*- to the *cis*-configuration (text-fig. 1).

The pathologic-anatomic picture found in the dead animals was practically identical following administration of different podophyllin derivatives of the A series. An increased blood supply was observed in most of the intestines; in addition, in the kidneys hemorrhaging into the glomeruli and parenchymatous degeneration were noted. But the increase of blood in the intestines did not reach the level described by Neuberger (*3*) in rabbits, dogs, and cats.

Significant transformations were observed in tissues which generally, even in fully grown animals, show increased mitotic activity. In skin many atypical mitoses, including stathmokineses, were seen (fig. 1), particularly in the basal layers of the epidermis and, locally, in the deeper portions of the epithelial sheath of the hair follicles. Furthermore, even in other types of epithelium (*i.e.*, epithelium of the cornea, gastric mucosa, crypts of Lieberkühn in the small intestine), in addition to normal mitoses similar atypical phenomena were observed. In the ground epithelium of the testicular canals (fig. 2) we observed a range of atypical forms, from the initial phases of division to stathmokineses, in cells in the spermatocyte stage; the other stages showed transformations of negligible significance.

In the liver we noted a large number of mitoses in the Kupfer cells and the previously mentioned increase in blood volume. The mitoses were either normal or partly damaged. In the liver cells, mitoses in the stage



Podophyllotoxin
trans(8:7)*trans*(7:6)*cis*(6:5)
biologically active

Picropodophyllin
trans(8:7)*cis*(7:6)*trans*(6:5)
biologically inactive

Epipodophyllotoxin
cis(8:7)*trans*(7:6)*cis*(6:5)
biologically inactive

Epipicropodophyllin
cis(8:7)*cis*(7:6)*trans*(6:5)
biologically inactive

TEXT-FIGURE 1.—Design of configuration of substituents of B ring.

of anaphase and metaphase were found only rarely (fig. 3); more often, cells in the reconstruction phase were observed. It follows, therefore, that podophyllin derivatives stimulate mitotic division, even in normal intact livers, and show a special affinity for the reticuloendothelial elements.

The foregoing facts suggest that even normal mitotic division in different organs of fully grown animals may be affected by podophyllin derivatives in a way analogous to the effect of colchicine derivatives (*28,29*).

Stathmokinetic Effect

The stathmokinetic effect, investigated in regenerating rat livers, differed somewhat in kind and extent from the effect produced by colchicine and its derivatives.

Qualitative evaluation.—The mitotic figures in sections of regenerating rat liver presented a rather varied picture following administration of podophyllin derivatives of the A series, although the individual toxic substances under investigation do not differ fundamentally (figs. 4–7).

In all cases with biologically active compounds the histologic findings showed slightly aberrant mitoses along with normal ones (fig. 7) in both the initial and final phases of division. The first effects of podophyllin substances that we found were the induction of mitotic arrest in metaphase (fig. 5) combined with the aggregation of chromosomes and chromatin substances in the center of the cells in the form of a clod or a ring. Atypical mitotic figures, in which larger or smaller particles of chromatin are detached from the otherwise normally arranged mitotic figure, occurred frequently. Comparatively large numbers of tripolar mitoses (fig. 4) were also observed. Stathmokineses, which did not differ at all from the typical stathmokineses seen with colchicine, represented another degree of damage.

Furthermore, we observed cells with many tiny, round "nuclei," which have been reported to be due to the effect of colchicine in plant material (*30,31*) and in animals (*32*); Kantner (*33*) reported these to be groups of karyomerites. In addition to the mitotic figures already discussed, in some cases we found formations about the size of a liver cell, or smaller ones containing eosinophilic plasma, which were filled with a great number of chromatin granules of different sizes that stained with varying intensity. They are evidently the products of mitoses damaged by podophyllin, for example, the karyomerites, which have been phagocytosed from the blood by the reticuloendothelial elements. This is confirmed by the fact that in some cases chromatin granules were observed free in the sinusoids of the liver (fig. 6).

All this suggests that administration of podophyllin substances produces many more variations in mitotic division than colchicine. The explanation may be that the podophyllin substances do not exercise such a radical influence on mitotic division. They are able to arrest it completely, producing stathmokineses and complete decomposition of the cells or to affect mitoses less intensely, thereby producing atypical mitoses, or in some cases they may be without effect entirely.

Quantitative evaluation.—Quantitatively, the greatest mitotic activity could be observed following administration of podophyllotoxin, which produced an effect very similar to that of colchicine, although, of course, after a sevenfold larger dose. The other toxic substances of the A series produced a lesser stathmokinetic effect. Doses approximating the LD50 on the whole had a rather mild effect, which increased only slightly with further increases in dose. The curve representing the stathmokinetic index does not show a steep rise, whereas such a rise was observed with colchicine and its derivatives and with one of the substances under investigation, *viz.*, podophyllotoxin. The stathmokinetic effect of the acetylated derivatives of the A-series is still lower, *i.e.*, on the whole, it parallels the decreasing toxicity of these substances. The derivatives of the B series did not affect mitotic division at all.

This observation suggests that podophyllotoxin, α-peltatin-A, and β-peltatin-A are less suitable for therapeutic use than colchicine derivatives, in many of which the stathmokinetic and lethal doses are widely divergent from each other. For example (*24*), in an analogous experiment with colchicine, the LD100 was 5 mg. per kg., the LD50, 2 mg. per kg., and the dose producing stathmokinesis was 2 mg. per kg. Substance F, or demecolcine, has an LD100 at 35 mg. per kg., an LD50 at 15 mg. per kg., and a stathmokinetic effect at 5 mg. per kg., so that the toxicity-stathmokinesis index (computed on the basis of LD100) is 2.5 for colchicine and 7 for demecolcine. But for all podophyllin derivatives investigated by us this index was found to range between 1.5 and 2.7.

As already mentioned in the section on toxicity, no stathmokinetic activity was noted with the derivatives of the B series, in which the lactone ring underwent a change from the *trans*- to the *cis*-configuration. Furthermore, no effectiveness was observed with epipodophyllotoxin, in which the hydroxyl group of the B ring changes from the *trans*- to the *cis*-configuration, or with podophyllotoxon, in which the hydroxyl group is transformed to a carbonyl group. In the same way, deoxypodophyllotoxin, without any hydroxyl group on the B ring, is found to be biologically inactive. Conversion of epipodophyllotoxin into the B series does not restore the biological effectiveness of this compound.

All derivatives mentioned thus far are insoluble in water. For this reason glycosidic derivatives of podophyllotoxin or the peltatins were sought, since their solubility would make them more suitable for therapeutic use. Except for picropodophyllin acetyl glucopyranosyl none of these derivatives were available to us, but according to Stoll, Renz, and Wartburg (*20*) the 1-O/β-D-glucopyranosyl-/-podophyllotoxin isolated by them produces considerably less biological activity than podophyllotoxin.

Relationship between Structure of Compounds and Biological Effectiveness

The results of the study suggest that for maintenance of the biological activity of the podophyllin derivatives it is particularly necessary to maintain the configuration of the substituents of the B ring, *i.e.*, the *trans*(8:7)*trans*(7:6)*cis*(6:5) configuration.

With the peltatins it is necessary to maintain at least the *trans*(7:6)*cis*-(6:5) configuration. With podophyllotoxin, the hydroxyl group of the B ring of these compounds is transferred to the A ring. It may be concluded that the presence of a free hydroxyl group on the A or B ring (*i.e.*, on C_1 or C_8) must be necessary for biological effectiveness, because neither deoxypodophyllotoxin, podophyllotoxon, nor the alkylated peltatins of the A series have been found to produce biological activity; the acylated derivatives that are able to deacetylate in the organism are considerably less active. When the hydroxyl group is situated on the B ring, it must be in the *trans*-configuration to the substituent at C_7, for epipodophyllotoxin, which has this hydroxyl group in the *cis*-configuration, is ineffective.

The demethylation of the methoxyl group on carbon 4′ (C ring) does not affect the decrease or increase of biological activity (4′-demethylpodophyllotoxin, α-peltatin-A).

Summary

1) A study of the LD50 and LD100 and the pathologic-anatomic effects in Wistar rats for podophyllotoxin, α-peltatin-A, and β-peltatin-A, demethylpodophyllotoxin, deoxypodophyllotoxin, epipodophyllotoxin, podophyllotoxon, methylated α-peltatin-A, and derivatives of the B series of the majority of the compounds mentioned above has been presented. The acetyl derivatives of some of the compounds were also investigated.

2) The stathmokinetic effect of the aforementioned substances, determined in regenerating rat livers, could be demonstrated, but the dose that produced it was very often equal to the LD50 or even the LD100.

3) The toxicity-stathmokinesis index of the podophyllin derivatives was about 1.5 to 2.7, whereas it was 2.5 for colchicine and 7 for demecolcine.

4) After studying the influence of structure on the biological activity of the podophyllin derivatives it was established that the compound must have the following properties:

a) A free hydroxyl group in position 1 or 8. Alkylation or acylation of this hydroxyl group lowers the effectiveness considerably (acylated derivatives) or causes its disappearance (alkylated derivatives).

b) The replacement of the hydroxyl group at C_8 for the carbonyl group, or its complete removal, is followed by the loss of biological activity.

c) The substituents in positions 5, 6, 7, and 8 should have the *trans*-(8:7)*trans*(7:6)*cis*(6:5) or at least the *trans*(7:6)*cis*(6:5) configuration. The change to the *trans*(8:7)*cis*(7:6)*trans*(6:5) configuration leads to the loss of biological activity.

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PLATE 29

FIGURE 1.—Epidermis of rat 28 hours after injection of 50 mg. per kg. of podophyllotoxin. *Note* occurrence of numerous damaged mitoses in the basal layer. One typical stathmokinesis is indicated by *arrow*. × 250

FIGURE 2.—Testis of same rat as in figure 1. Apart from normal mitotic formations, there are numerous damaged mitoses, including stathmokineses. × 250

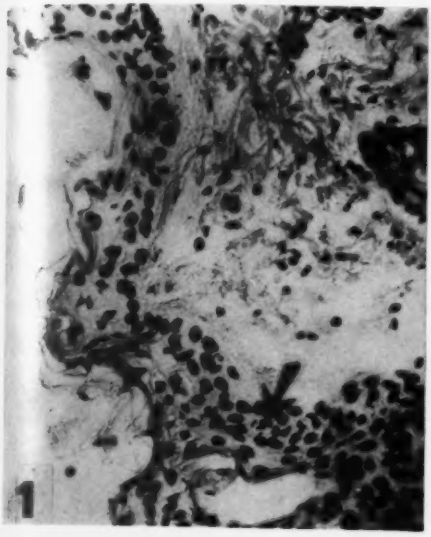
FIGURE 3.—Anaphase in normal (unregenerating) rat livers 36 hours after administration of 100 mg. per kg. of acetylated α-peltatin-A. × 490

FIGURE 4.—Tripolar mitosis in regenerating rat livers 18 hours after administration of 15 mg. per kg. of α-peltatin-A. × 540

FIGURE 5.—Two different stages of the genesis of the stathmokineses. At *left*, mitosis arrested in metaphase with chromosomes aggregating in the center of the cell. At *right*, single chromosomes and their particles begin to spread throughout cell. Same rat as in figure 4. × 540

FIGURE 6.—Aggregations of irregular chromatin granules and cell detritus in the sinusoids of the liver. Regenerating rat livers 18 hours after injection of 15 mg. per kg. of podophyllotoxin. × 540

FIGURE 7.—Stathmokinetic effect of podophyllotoxin in the rat shown in figure 6. Apart from numerous stathmokineses, on *left*, normal mitoses are found locally (1 normal telophase). × 540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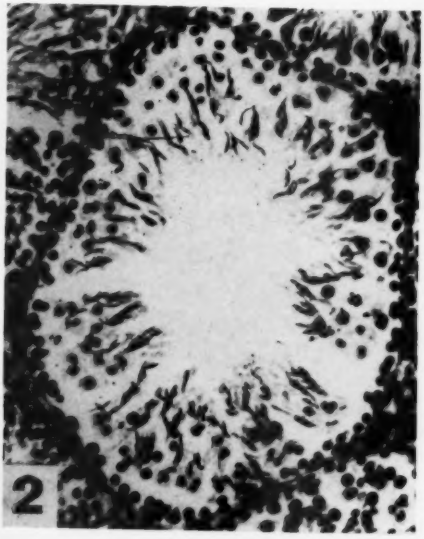
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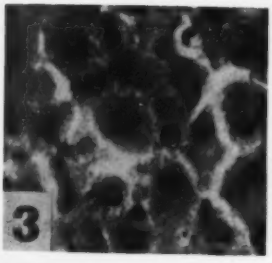
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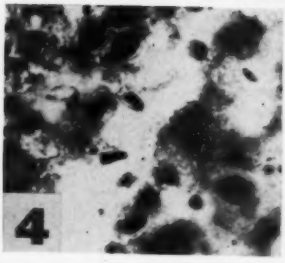
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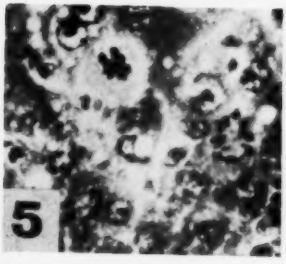
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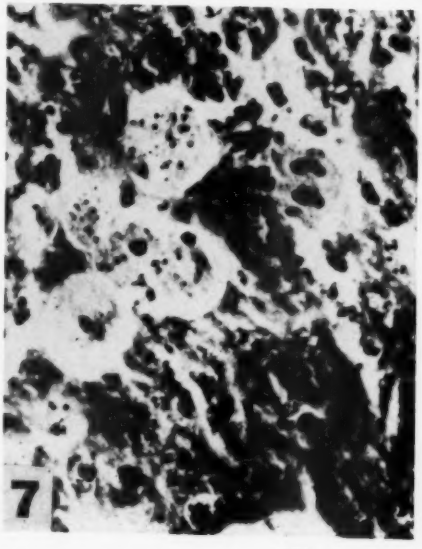
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Cancer Morbidity in Miyagi Prefecture, Japan, and a Comparison with Morbidity in the United States [1]

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A survey of the morbidity from cancer in a defined geographic area has been needed for a long time in Japan; yet, to our regret, it has not been performed. In response to the growing demand, the first survey in terms of cancer morbidity covering the residential population in a definite area was carried out in Miyagi Prefecture for the years 1951 to 1953. The outline of our study will be reported in this paper, followed by a comparison of the pattern of cancer morbidity in Miyagi Prefecture with that in the United States.

Miyagi Prefecture, which is one of the 46 prefectures in Japan, is located in the northeastern part on the Pacific coast of Honshu, the mainland of Japan. It has a population of 1,727,044 (enumerated as of the 1955 population census) and an area of 7,273 square kilometers. Sendai City is the capital of the prefecture and the cultural and administrative center, where Tohoku University Hospital is located. This hospital is the largest not only in Miyagi Prefecture but also in the adjacent 5 prefectures, which cover what we call "Tohoku District," the northeastern district of Honshu. Accordingly, many patients among the residents of the neighboring prefectures as well as those of Miyagi Prefecture tend to come to the Tohoku University Hospital, whereas the number of patients among the residents of Miyagi Prefecture who go out to the hospitals in the neighboring prefectures is assumed to be small.

In our survey, all the cancer cases, treated or observed by all physicians working at the hospitals and the medical offices [3] in Miyagi Prefecture in 1951, 1952, and 1953, including the cases discovered during visits to homes, were enumerated. In designing this survey, we were guided to a great extent by the methods used for the cancer register in Denmark (*1–3*) and the survey of the 10 metropolitan areas of the United States (*4–6*).

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[3] In Japan, a "hospital" is defined as a medical institution with 20 or more beds and with equipment and personnel that meet the requirements regulated by law, whereas medical institutions other than hospitals are called "medical offices." In 1954, the number of hospitals and medical offices in Miyagi Prefecture was 106 and 837, respectively.

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Method

Collection of data.—Data concerning all patients with cancer who were being treated or observed in all the hospitals and medical offices during 1951, 1952, and 1953, were collected in April, 1952, June, 1953, and January, 1955, respectively. For each patient the following information was requested: name, address, sex, age, date of first subjective symptom, clinical diagnosis, including site of cancer development, and method of confirmation of disease (microscopically, by surgery, by X-ray examination, other). These cases were coded in accordance with the sixth revision of the *International List of Diseases and Causes of Death* (7). In addition, a death-certificate search for persons who had died of cancer was carried out in each of the 15 local health centers of Miyagi, where these certificates are filed.

The number of cancer cases among residents in the Prefecture, including deaths, prevalent in each study year is given in table 1. The enumeration was done by carefully checking the name and address of each case and the elimination of all duplications. As shown in table 2, the number of cases prevalent during the 3 study years 1951 to 1953 was 6,574.

TABLE 1.—*Number of cancer cases, including deaths, prevalent in each study year: 1951, 1952, and 1953*

Cases	1951	1952	1953
Total	2, 297	2, 718	2, 669
Reported by physicians	1, 529	2, 088	1, 907
Checked on death certificates only	768	630	762

Definition of cancer incidence.—From the standpoint of morbidity there is a question about what criterion should be accepted for establishing the date of onset. Although it is desirable to study each case thoroughly to establish a definite date of onset, such detailed reviews were not practical for this survey. We could not consider the date of first diagnosis as the date of onset, as was done in the survey of the 10 metropolitan areas, because patients customarily see several physicians and are usually not told that they have cancer. Thus, it was not possible for a physician to know whether a patient had cancer that had been diagnosed previously. Therefore, we did not request physicians to report previous diagnoses of cancer among their patients.

In this survey, we decided to consider date of onset as the time that the patient complained of his first symptom connected with the malignancy. Thus, for example, some were excluded from the data for 1951 if the first subjective symptoms had appeared during 1950 or before. Some reported in 1952 were tabulated in the 1951 group if they had claimed their first subjective symptoms during 1951. We collected all the records of the patients under the care of physicians during 1951, 1952, and 1953. However, the 1953 results will not be complete until we have the data of 1954 for the above reasons.

There were a considerable number of cancer deaths that were checked only by death certificates, which included information on the date of death and the interval between onset and death. To compute the date of onset we counted back from the date of death the number of days during the interval between onset and death.

Results

Number of cases.—On the basis of reports from physicians and death certificates, the number of cancer cases prevalent during the 3 study years 1951 to 1953 among residents in Miyagi Prefecture proved to be 6,574, which did not include the nonresident cases (table 2). Of these 6,574 cases there were 1,417 who had had their first subjective symptoms in the years prior to 1951 and were, therefore, excluded from the tabulation for the 3 study years. On the other hand, the figures on cancer incidence for the year 1953 are to be revised upward later, based upon data collected in subsequent years, for reasons mentioned previously. However, the data for the years 1951 and 1952 can be considered almost complete,

TABLE 2.—*Number of cancer cases whose first subjective symptom appeared during the study years 1951, 1952, and 1953 or prior to 1951*

Year first symptom appeared	Cases	Both sexes	Male	Female
	Total	6, 574	3, 182	3, 392
1953	Total	1, 319	656	663
	Reported by physicians	1, 060	536	524
	Checked on death certificates	259	120	139
1952	Total	1, 929	966	963
	Reported by physicians	1, 432	702	730
	Checked on death certificates	497	264	233
1951	Total	1, 909	953	956
	Reported by physicians	1, 327	650	677
	Checked on death certificates	582	303	279
Before 1951	Total	1, 417	607	810
	Reported by physicians	916	372	544
	Checked on death certificates	501	235	266

even though there might be a small number of cases to be added later on. The number of cancer cases for the years 1951 and 1952, based on the date of the first subjective symptom, was 1,909 and 1,929, respectively; of the total of 3,838 for these 2 years, exactly half were males and the other half females. Further analyses of morbidity data are presented for the years 1951 and 1952 only, because we consider these to be the more reliable at present.

Accuracy of diagnosis.—We checked the method of diagnosis, for 1,515 of 1,529 cases reported during the year 1951, by referring to the records

sent by physicians in response to our inquiries. These records were classified into the following categories: *a*) microscopic examination explicitly reported; *b*) surgery indicated, but no record of microscopic examination; *c*) X-ray examination, but no record of microscopic examination or surgery; *d*) others. Each of the preceding categories was subdivided according to site of cancer, as indicated in table 3. The result revealed that, of the total of 1,515 cases, only 28 percent of the diagnoses had been confirmed by microscopic examination, at least on record. We guess that the percentages for those actually diagnosed by microscopic confirmation were somewhat higher than the figures given in the table. In any case, we admit that the percentage is not high enough to assure perfect reliability. In this respect, we are aware that our data, strictly speaking, are not entirely accurate.

TABLE 3.—*Number of reported cancer cases confirmed by microscopic examination, surgery, or X ray, by primary site, for 1951*

Site	No. cases reported	Microscopic confirmation		Surgery but no mention of microscopic confirmation*		X-ray confirmation only	
		Number	Percent	Number	Percent	Number	Percent
Total	1,515	425	28.1	352	23.2	323	21.3
Carcinoma							
Buccal cavity and pharynx	17	9	52.9	1	5.9	—	0
Digestive system	851	134	15.7	195	22.9	294	34.5
Esophagus	69	11	15.9	3	4.3	39	56.5
Stomach	598	99	16.6	157	26.3	213	35.6
Intestines	24	9	37.4	4	16.7	1	4.2
Rectum	61	13	21.4	20	32.8	4	6.5
Liver and biliary passages	78	2	2.6	6	7.2	27	34.8
Pancreas	17	—	0	3	17.7	10	58.9
Peritoneum	4	—	0	2	50.0	—	0
Respiratory system†	32	12	37.6	1	3.1	12	37.6
Accessory sinuses	23	6	26.0	3	13.0	4	17.4
Breast	105	33	31.4	54	51.4	—	0
Uterus‡	257	111	43.2	66	25.7	—	0
Other female genital organs§	35	11	31.4	10	28.6	3	8.6
Male genital organs	14	8	57.0	1	7.1	—	0
Urinary organs	11	3	27.4	2	18.2	3	27.4
Skin	16	7	43.6	3	18.7	—	0
Thyroid gland	15	11	73.4	2	13.3	—	0
Other and unspecified sites	19	7	36.8	2	10.5	3	15.8
Malignant neoplasms of eye	7	6	86.0	—	0	—	0
Malignant neoplasms of brain and nervous system	5	4	80.0	—	0	—	0
Lymphomas	37	22	59.4	1	2.7	—	0
Hodgkin's disease	6	4	66.7	2	33.3	—	0
Leukemias and aleukemias	17	14	82.4	—	0	—	0
Sarcoma‖	40	20	50.0	8	20.0	2	5.0
Other and unspecified malignant neoplasms	8	3	37.6	1	12.5	1	12.5

*Some of the surgical cases may have had microscopic confirmation.
†Excluding accessory sinuses.
‡Excluding chorio-epithelioma.
§Including chorio-epithelioma.
‖Including all sites.

Variation in cancer incidence by sex and primary site.—The number of cancer cases among the residents of Miyagi Prefecture during 1951 and 1952, classified by sex and primary site, is indicated in table 4. From the figures given in the table, the rate of cancer incidence for all sites for these 2 years was 116.2 in males and 115.3 in females per 100,000 persons of each sex, respectively.

It was observed that, in males, the site of most frequent occurrence was the digestive organs and peritoneum, with a total of 1,464 cases

TABLE 4.—*Number of cases and number of deaths from cancer, by sex and primary site, for 1951 and 1952*

Site	Number of cases		Number of deaths	
	Male	Female	Male	Female
All cancers	1,919	1,919	1,455	1,300
Buccal cavity and pharynx	16	17	12	11
Lip	1	1	3	—
Tongue	8	7	5	2
Salivary gland	2	2	1	1
Mouth	1	—	1	—
Pharynx	4	7	2	8
Digestive organs and peritoneum	1,464	902	1,142	730
Esophagus	153	63	121	59
Stomach	1,022	570	789	451
Small intestine	1	3	2	3
Large intestine, except rectum	41	46	25	33
Rectum	56	62	40	45
Liver	131	101	116	100
Biliary passages	37	25	31	16
Pancreas	20	24	13	13
Peritoneum	3	8	3	10
Unspecified digestive organs	—	—	2	—
Respiratory system	105	60	71	39
Accessory sinuses	27	24	15	13
Nose, nasal cavity, and middle ear	2	6	—	—
Larynx	23	9	17	4
Trachea, bronchus, and lung	45	16	35	18
Pleura	3	2	2	2
Mediastinum and other thoracic organs	5	3	2	2
Breast	3	154	1	56
Genital organs	22	516	10	283
Cervix uteri		297		121
Corpus uteri		15		6
Other and unspecified parts of uterus		147		126
Chorio-epithelioma		34		15
Ovary, fallopian tube, and broad ligament (all cases ovary)		15		13
Other and unspecified female genital organs		8		2
Prostate	11		6	
Testis	7		1	
Other and unspecified male genital organs	4		3	
Urinary organs	15	16	15	9
Kidney	5	2	4	2
Bladder	10	13	11	5
Other urinary organs	—	1	—	2

TABLE 4.—Continued

Site	Number of cases		Number of deaths	
	Male	Female	Male	Female
Skin	11	15	5	9
Eye	9	3	2	3
Brain and nervous system (including unspecified nature)	32	28	28	29
Thyroid gland	4	20	3	4
Other endrocrine glands	1	—	1	—
Bone	12	13	7	7
Connective tissue	2	2	—	1
Other and unspecified sites	26	27	18	20
Neoplasms of lymphatic and hematopoietic tissues	101	74	81	57
Lymphosarcoma and reticulosarcoma	40	34	30	21
Hodgkin's disease	18	7	11	7
Leukemia and aleukemia	43	33	40	29
Reticulosis, multiple myeloma, and mycosis fungoides	—	—	—	—
Neoplasms of unspecified nature	95	70	59	42
Complication cases of 2 kinds of primary cancer	1	2	—	—

(76.3% of all cancers); of these, 1,022 (53.3%) were gastric cancers. For the respiratory system the total was 105 (5.5%). Leukemia and aleukemia, lymphosarcoma and reticulosarcoma, and Hodgkin's disease were included in the category of neoplasms of lymphatic and hematopoietic tissues, with an aggregate of 101 cases (5.5%).

In females, cancer of the digestive organs and peritoneum was observed in 902 cases (47.0%), of which 570 (29.7%) were gastric cancers. The number of cases of cancer of the female genital organs was 516 (26.9%). Most of the cases of uterine cancer, a site for which there were inaccurate listings in the physicians' records, are considered to have originated in the cervix uteri. Clinical statistical data provided by the hospitals in Japan reveal that, in Japan, the frequency of cancer of the corpus uteri is very low compared with that of cervical cancer. As a matter of fact, according to the author's data, obtained at the Department of Obstetrics and Gynecology of Tokyo University Medical School, of 2,461 uterine-cancer cases examined, only 77 (3.1%) were cancer of the corpus uteri. The figures reported in other Japanese clinical statistical data range from 3 to 5 percent. Taking such evidence into consideration, I would like to mention here that, in Japan, when "cancer of the uterus" appears as a diagnosis in physicians' records, it usually means "cervical cancer." The number of breast-cancer cases among females was 154, or 8.0 percent of all cases. Neoplasms of lymphatic and hematopoietic tissues were observed in 74 cases (3.9%).

Variation in rate of cancer incidence by site, age, and sex.—The cancer-incidence rate by site, age, and sex is presented in table 5. The pattern of age and sex distribution for each cancer site is self-explanatory in the table.

Comparison of Cancer Incidence in Miyagi Prefecture with That in 10 Metropolitan Areas of the United States Based on Age-Adjusted Rates

For the purpose of comparing our cancer-incidence data with those in the 10 metropolitan areas of the United States, our figures were adjusted according to the age composition of the United States white population in these areas. We then computed the ratio of the adjusted Japanese figures to those for the United States. The United States data quoted in this paper are those in the monograph published by Dorn and Cutler (*6*). As noted in a previous paragraph, our criterion for computing incidence is different from that used in the United States. Nevertheless, we believe that a rough comparison of the two sets of incidence rates is practicable. Table 6 represents the results of our computation. For convenience of description, Miyagi Prefecture and the 10 metropolitan areas in the United States will be designated as "Japan" and "U. S.," respectively.

In the U. S. the cancer-incidence rate per 100,000 for all sites is 324.4 in males and 347.7 in females, whereas the rate in Japan is 199.6 in males and 178.8 in females. Observing the ratios of the Japanese rates to those of the U. S., 61.5 in males and 51.4 in females on the basis of 100 for each sex in the U. S. rates, we learn that the incidence of cancer is, in general, remarkably low in Japan compared with that in the United States.

When the figures are analyzed by site and sex, several outstanding differences between the Japanese and U. S. rates are noted. The frequency of cancer of the buccal cavity and pharynx is much lower in Japan than in the U. S., whereas that of the esophagus, stomach, and liver and biliary passages is considerably higher in Japan. It is remarkable that, in Japan, the incidence of cancer of the trachea, bronchus, and lung is much lower compared with that in the U. S. A similar tendency is observed for breast cancer in females. As for uterine cancer, the frequency, in terms of rate of incidence, is lower in Japan. There is a tremendous difference between the incidence of ovarian cancer in Japan and the U. S. That is, the U. S. rate is about 15 times as great as the Japanese. Cancer of the male genital organs is also very infrequent among the Japanese. As a matter of fact, the incidence rate for cancer of the prostate in the U. S. is about 25 times as great as that in Japan. A similar relationship is observed for cancer of the urinary organs and for cancer of the skin. As shown in table 6, the indices of the incidence rates for skin cancer in Japan are only 1.8 in males and 3.2 in females, when the U. S. rates are equal to 100. The incidence of neoplasms of the eye, brain, and other parts of the nervous system as well as that of lymphatic and hematopoietic tissues is also much higher in the U. S.

Summary

Results of a survey of the incidence of cancer in Miyagi Prefecture, Japan, during a 3-year period are presented. This study is the first to deal with a circumscribed geographic area in Japan. A comparison of

TABLE 5.—*Cancer-incidence rates per 100,000 population, by sex, age, and primary site, for 1951 and 1952*

Site	Sex	All ages	<10	10–19	20–29	30–39	40–49	50–59	60–69	70–79	80+
All cancers	M	116.2	11.3	7.0	18.3	39.2	149.4	409.3	952.6	1331.7	829.0
	F	115.3	8.8	8.1	21.0	83.7	229.8	406.1	644.6	727.2	531.5
Buccal cavity and pharynx	M	1.0	0	0	0	0	2.0	2.7	12.8	4.0	0
	F	1.0	0	0	0.3	0.5	0.7	1.8	10.1	10.7	0
Digestive organs and peritoneum	M	88.7	0	0.3	4.7	21.8	114.7	345.3	794.8	1083.0	571.8
	F	54.2	0	0.8	5.1	19.7	75.9	176.8	399.9	504.5	358.8
Esophagus	M	9.3	0	0	0.3	1.7	4.0	37.4	75.4	180.5	85.8
	F	3.8	0	0	0.3	0.5	2.6	9.1	30.3	53.7	39.9
Stomach	M	61.9	0	0.3	3.4	12.3	86.7	247.4	570.1	677.9	285.9
	F	34.2	0	0.3	3.5	14.8	50.9	112.4	251.0	295.2	225.9
Large intestine	M	2.5	0	0	0.3	4.5	2.0	10.7	14.2	28.1	0
	F	2.8	0	0	0	1.0	2.6	10.9	22.7	26.8	0
Rectum	M	3.4	0	0	0.3	1.1	5.3	10.7	35.5	32.1	0
	F	3.7	0	0.3	0.7	2.0	7.3	10.0	25.2	29.5	26.6
Liver and biliary passages	M	10.2	0	0	0.3	2.2	12.7	32.0	89.6	152.4	200.1
	F	7.6	0	0	0	0.5	7.3	27.2	56.8	91.2	66.4
Other digestive organs	M	1.5	0	0	0	0	4.0	7.1	10.0	12.0	0
	F	2.1	0	0.3	0.7	1.0	5.3	7.3	13.9	8.0	0
Respiratory system	M	6.4	0.2	0	1.0	0.6	11.3	16.9	51.2	92.3	114.4
	F	3.6	0	0.5	1.0	1.0	7.3	13.6	18.9	26.8	26.6
Larynx	M	1.4	0	0	0	0	2.0	3.6	12.8	20.1	57.2
	F	0.5	0	0	0	0.5	0.7	0.9	5.0	5.4	0
Trachea, bronchus, and lung	M	2.7	0	0	0	0	3.3	8.0	25.6	44.1	28.6
	F	1.0	0	0	0.3	0	1.3	4.5	6.3	8.0	0
Other respiratory organs	M	2.2	0.2	0	1.0	0.6	6.0	5.3	12.8	28.1	28.6
	F	2.1	0	0.5	0.3	1.5	4.6	8.2	7.6	13.4	26.6
Breast	M	0.2	0	0	0	0	1.3	0	0	4.0	0
	F	9.3	0	0	2.1	13.8	28.4	42.6	22.7	29.5	13.3

Genital organs	M	1.3	0.5	0	0	1.1	2.0	4.4	5.7	20.1	28.6
	F	31.0	0.2	0.5	4.8	33.0	95.1	136.9	128.7	75.1	79.7
Uterus	F	29.6	0	0	3.8	32.0	91.8	130.5	126.2	72.4	79.7
Ovary	F	0.9	0.2	0.5	1.0	1.0	2.6	1.8	1.3	0	0
Other female genital organs	F	0.5	0	0	0	0	0.7	4.5	1.3	2.7	0
Prostate	M	0.7	0	0	0	0	0.7	2.7	4.3	16.0	0
Other male genital organs	M	0.7	0.5	0	0	1.1	1.3	1.8	1.4	4.0	28.6
Urinary organs	M	0.9	0.5	0	0	0	0	4.4	4.3	16.0	28.6
	F	1.0	0.2	0	0	0	2.0	1.8	7.6	10.7	0
Skin	M	0.7	0	0	0.7	0.6	1.3	0.9	4.3	8.0	0
	F	0.9	0.2	0	0	1.0	2.0	0.9	7.6	5.4	0
Eye, brain, and nervous system	M	2.5	3.5	0.3	3.4	2.8	1.3	3.6	2.8	4.0	0
	F	1.9	2.1	1.3	1.0	2.5	2.6	2.7	2.5	0	0
Endocrine organs	M	0.3	0	0	0	0	0.7	1.8	2.8	0	0
	F	1.2	0	0	0.3	1.5	4.0	2.7	5.0	5.4	13.3
Bone	M	0.7	0	0.8	0.3	0.6	2.0	1.8	1.4	4.0	0
	F	0.8	0.2	1.1	0.7	0	0.7	1.8	3.8	0	0
Connective tissue	M	0.1	0	0.3	0	0	0.7	0	0	0	0
	F	0.1	0	0.3	0	0	0.7	0	0	0	0
Other and unspecified sites	M	1.6	0.9	0	0	0.6	0	2.7	10.0	36.1	57.2
	F	1.6	0.5	0.8	0.7	2.0	0	5.4	6.3	10.7	13.3
Lymphatic and hematopoietic tissues	M	6.1	3.7	4.6	4.1	3.9	4.7	13.3	27.0	24.1	28.6
	F	4.4	4.1	2.4	2.8	4.4	7.3	7.3	11.4	5.4	13.3
Lymphomas	M	3.5	1.2	2.3	2.4	1.7	3.3	10.7	19.9	12.0	0
	F	2.5	0.7	0.3	1.7	2.5	6.6	5.4	10.1	5.4	13.3
Leukemia and aleukemia	M	2.6	2.5	2.3	1.7	2.2	1.3	2.7	7.1	12.0	28.6
	F	2.0	3.3	2.2	1.0	2.0	0.7	1.8	1.3	0	0
Neoplasm of unspecified nature	M	5.8	2.1	0.8	4.1	7.3	7.3	10.7	35.5	36.1	0
	F	4.2	1.0	0.3	2.1	3.9	3.3	11.8	20.2	40.2	13.3
Complication cases of 2 kinds of primary cancer	M	0.1	0	0	0	0	0	0.9	0	0	0
	F	0.1	0	0	0	0.5	0	0	0	2.7	0

TABLE 6.—*Comparison of age-adjusted cancer-incidence rates, by site and sex, in Miyagi Prefecture and 10 metropolitan areas in the U. S.**

Site	Japan		United States		Japan† / United States	
	Male	Female	Male	Female	Male	Female
All cancers	199.6	178.8	324.4	347.7	61.5	51.4
Buccal cavity and pharynx	1.8	1.6	20.6	6.5	8.7	24.6
Digestive organs and peritoneum	158.1	87.0	105.5	81.9	149.9	106.2
Esophagus	17.0	6.2	7.9	1.9	215.2	326.3
Stomach	109.6	54.4	32.3	18.6	339.3	292.5
Large intestine	4.3	4.4	24.8	28.7	17.3	15.3
Rectum	5.9	5.9	21.3	15.7	27.7	37.6
Liver and biliary passages	18.7	12.5	7.0	8.8	267.1	142.0
Other digestive organs	2.5	3.2	12.2	8.2	20.5	39.0
Respiratory system	11.3	5.6	39.5	8.6	28.6	65.1
Larynx	2.7	0.9	7.7	0.7	35.1	128.6
Trachea, bronchus, and lung	4.9	1.5	30.4	6.7	16.1	22.4
Other respiratory organs	3.8	3.3	1.4	1.2	271.4	275.0
Breast	0.3	14.4	0.8	76.6	37.5	18.8
Genital organs	2.3	48.6	33.0	79.1	7.0	61.4
Uterus		46.7		60.1		77.7
Ovary		1.1		15.6		7.1
Other female genital organs		0.8		3.4		23.5
Prostate	1.2		29.2		4.1	
Other male genital organs	1.1		3.8		28.9	
Urinary organs	1.6	1.5	23.3	11.7	6.9	12.8
Skin	1.0	1.3	54.3	40.9	1.8	3.2
Eye, brain, and nervous system	2.6	1.9	7.5	5.1	34.7	37.3
Endocrine organs	0.5	1.9	2.0	4.1	25.0	46.3
Bone	1.0	0.9	2.2	1.9	45.5	47.4
Connective tissue	0.1	0.1	2.1	1.9	4.8	5.3
Lymphatic and hematopoietic tissues	7.7	5.2	19.7	14.2	39.1	36.6
Lymphomas	4.8	3.5	11.0	7.9	43.6	44.3
Leukemia and aleukemia	3.0	1.7	8.7	6.3	34.5	27.0
Other and unspecified sites	2.8	2.2	13.9	15.2	20.1	14.5

*Rates per 100,000 population. Rates for Miyagi Prefecture are adjusted on the basis of the age distribution of the white population in 10 metropolitan areas of the U. S. in 1947.

†Denotes indices of the incidence rates of Japan, where the U. S. rates are equal to 100.

Japanese age-adjusted rates with United States rates for the white population of 10 metropolitan areas is included.

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Recorded and Expected Mortality among the Indians of the United States with Special Reference to Cancer [1]

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A deficit in mortality attributable to cancer among American Indians compared with cancer death rates for the white and nonwhite populations of the United States has been demonstrated by the Bureau of Indian Affairs (*1,2*). These analyses indicated that mortality ascribed to malignancies of the breast and to the lymphoma group was exceptionally low, being only one fifth as high as the rates among the white population. Hoffman (*3*) previously reported the relative infrequency of cancer among the Indians. The author and his associates (*4*) have demonstrated a deficit in mortality from cancer among the Navajo Indians, particularly cancer of the breast, prostate, and lung.

The purpose of this paper is to analyze the data on Indian mortality in greater detail than has been given for any Indian populations in previous studies, and to determine separately, for each sex, which specific primary sites were chiefly responsible for the observed deficit in all cancer deaths and whether excesses existed for any site. This was done by comparing the number of deaths attributed to cancer to the number expected to occur among the Indians had they been subject to the same rates prevailing among the white and nonwhite United States populations. Since the confidence which may be placed in differences demonstrated is to some extent influenced by the forces of mortality that replace or are replaced by cancer, comparisons have also been made for all causes of death and for deaths grouped in conventional broad causes.

The American Indian Population

Hadley (*5,6*) discusses the difficulties in determining the correct base population for which vital data relating to Indians are reported to the National Office of Vital Statistics. It is known that the 1950 Census represents an underenumeration of Indians (*5,7*). In the 1950 Census, an estimated 30,000 members of certain groups of mixed ancestry in the Southeast, previously included in the Indian group, were arbitrarily transferred to the residual category "other races." In addition, in areas such

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as Oklahoma, where Indians live among the general population, there is a tendency toward underenumeration of persons of part-Indian ancestry. Hadley (*5*) presents evidence indicating that vital events are reported to the National Office of Vital Statistics on a sizable but unknown proportion of these "Indians." He concludes that the base for calculating birth and death rates among Indians of the United States should be an "indeterminate" population between the census enumeration and those known to have legal rights as Indians. On the basis of data available from several sources, the estimated base population as of January 1, 1951 was 428,225. Since the age and sex distribution of this estimated population was not known, the distribution was assumed to be as in the 1950 Census. All subsequent analyses employ this estimate in the computation of expected deaths.

The majority of American Indians are entitled to medical care in hospitals and clinics maintained by the Federal Government or in facilities where care is furnished to them under Government contract. In addition, some areas have medical facilities supported by missions. Any Indian who so desires may seek medical care from private physicians and privately operated clinics and hospitals. A thorough study has been completed by the Public Health Service on "Health Services for American Indians" and a multilithed report bearing this title has been prepared.

Method of Study

The National Office of Vital Statistics furnished special tabulations of deaths from all causes among the Indians of the United States during the calendar years 1949–52, inclusive. Mortality among the Indians for all causes and for conventional broad causes, as well as those attributed to cancer of specific sites, was compared to deaths expected among them for the same period if they had been subject to the age-, sex-, and cause-specific death rates of the United States whites and nonwhites for 1950 and 1951.

The ratio of observed deaths to expected deaths and the ratio of the Indian average annual death rate, adjusted for age by the indirect method, to the United States death rate are the same. This ratio, multiplied by 100, is referred to as the standardized mortality ratio (SMR). The reasons for using the indirect method of age adjustment for analysis of mortality in relatively small populations have been discussed previously (*4,8*).

General Results

The results of computations comparing observed deaths with deaths expected on the basis of white or nonwhite mortality for all causes and for broad causes are shown in table 1. Indian average annual mortality rates for all causes are considerably higher than those of the whites, the differences being greater among females. The Indian over-all mortality is fairly comparable to that of the nonwhites and, in view of the uncertain Indian population base, little importance should be attached to the relatively small,

TABLE 1.—*Deaths recorded among Indians of the United States during 1949–52, inclusive, by broad causes, deaths expected on the basis of age-specific rates observed in 1950 and 1951 among the whites and nonwhites of the United States, and resultant standardized mortality ratios (SMR's)*

Broad cause of death	International list number	Males					Females				
		Number of deaths recorded	On basis of nonwhite rates		On basis of white rates		Number of deaths recorded	On basis of nonwhite rates		On basis of white rates	
			Number of deaths expected	SMR	Number of deaths expected	SMR		Number of deaths expected	SMR	Number of deaths expected	SMR
I Infective and parasitic	(001–138)	1, 222	827	148*	244	501*	1, 323	493	268*	123	1, 076*
II *Neoplasm*	(*140–239*)	*481*	*853*	*56**	*919*	*52**	*567*	*770*	*74**	*714*	*79**
Malignant	(140–205)	461	832	55*	899	51*	549	724	76*	692	79*
Benign	(210–239)	20	21	95	20	105	18	46	39*	22	82
III Allergy, endocrine, etc	(240–289)	130	136	96	125	104	177	178	99	131	135*
IV Blood-forming organs	(290–299)	26	29	90	24	108	29	27	107	21	138
V Mental diseases	(300–326)	77	46	167*	25	308*	37	21	176*	10	370*
VI Nervous and sensory	(330–398)	554	1, 089	51*	699	79*	447	972	46*	558	80*
VII Circulatory system	(400–468)	1, 642	3, 014	54*	2, 835	58*	1, 126	2, 218	51*	1, 570	72*
VIII Respiratory system	(470–527)	1, 069	680	157*	322	332*	944	490	193*	200	472*
IX Digestive system	(530–587)	785	390	201*	285	275*	749	260	288*	167	448*
X Genitourinary	(590–637)	227	440	52*	197	115†	158	284	56*	97	163*
XI Pregnancy and puerperium	(640–689)	—	—	—	—	—	128	94	136*	20	640*
XII Skin and cellular tissue	(690–716)	21	10	210*	5	420*	15	12	125	5	300*
XIII Bones and organs of movement	(720–749)	12	16	75	12	100	17	16	106	12	142
XIV Congenital malformation	(750–759)	144	127	113	154	94	104	96	108	131	79†
XV Early infancy	(760–776)	737	850	87*	528	140*	558	636	88*	365	153*
XVI Senility and ill-defined	(780–796)	884	506	175*	89	993*	767	368	208*	53	1, 447*
XVII Violence	(800–999)	1, 924	1, 283	150*	811	237*	678	435	156*	284	239*
All causes	(001–999)	9, 935	10, 296	96*	7, 274	137*	7, 824	7, 370	106*	4, 461	175*

*P=<0.01.
†*P*=0.01 to 0.05.

though statistically significant, differences noted. However, when broad causes of death are considered, Indian mortality for both sexes is materially and significantly higher than that of either the white or nonwhite populations for: infective and parasitic diseases (I), mental diseases (V), diseases of the respiratory system (VIII), diseases of the digestive system (IX), diseases of the skin and cellular tissue (XII), diseases attributed to senility and ill-defined causes (XVI), mortality from violence (XVII), and, for females, mortality due to complications of pregnancy and the puerperium (XI). On the other hand, the Indian rates are lower than the white or nonwhite in both sexes for: neoplasms (II), diseases of the nervous system and sense organs (VI), and diseases of the circulatory system (VII). For diseases of the genitourinary system (X) and diseases of early infancy (XV), Indian mortality for both sexes is higher than that of the white population and lower than that of the nonwhite. For the remaining broad causes little difference is noted, except that mortality attributed to allergies, endocrine, metabolic, and nutritional diseases (III) is higher among Indian females than white females.

Observed and Expected Mortality Attributed to Cancer

Text-figure 1 graphically presents the SMR's for sites in which the American Indians had interesting differences in mortality compared to white or nonwhite rates. The detailed data from which the text figure is derived are given in table 2. It is readily seen that Indian cancer mortality for both sexes is significantly lower than that among either whites or nonwhites. Furthermore, there is no site for which Indian males have higher mortality rates than the white or nonwhite males and only 3 sites in which Indian female rates are higher, namely, liver and pancreas, compared to both white and nonwhite rates, and cervix, compared to white rates only. For pancreas, however, the difference is significant only when compared to nonwhite female mortality and then only at the 5 percent level.

Among Indian males significant mortality deficits were found when comparisons were made with both white and nonwhite rates for cancer of the oral cavity, esophagus, intestines, rectum, lungs, prostate, urinary organs, and for the lymphoma group. In addition, when compared with mortality among white males, Indians also had significantly lower rates for malignant neoplasms of the skin, pancreas, brain, and nervous system, and for leukemia.

The standardized mortality ratios for cancer of the stomach, intestines, rectum, breast, and for the lymphoma group are materially and significantly lower in Indian females as compared with those for either whites or nonwhites. Mortality ascribed to malignancy of the ovary and to leukemia is significantly lower in Indian females than in white females. The mortality rates of Indian females are materially and significantly higher for cancer of the cervix than those of white females, while the death rate is nearly the same as the nonwhite rate. On the other hand,

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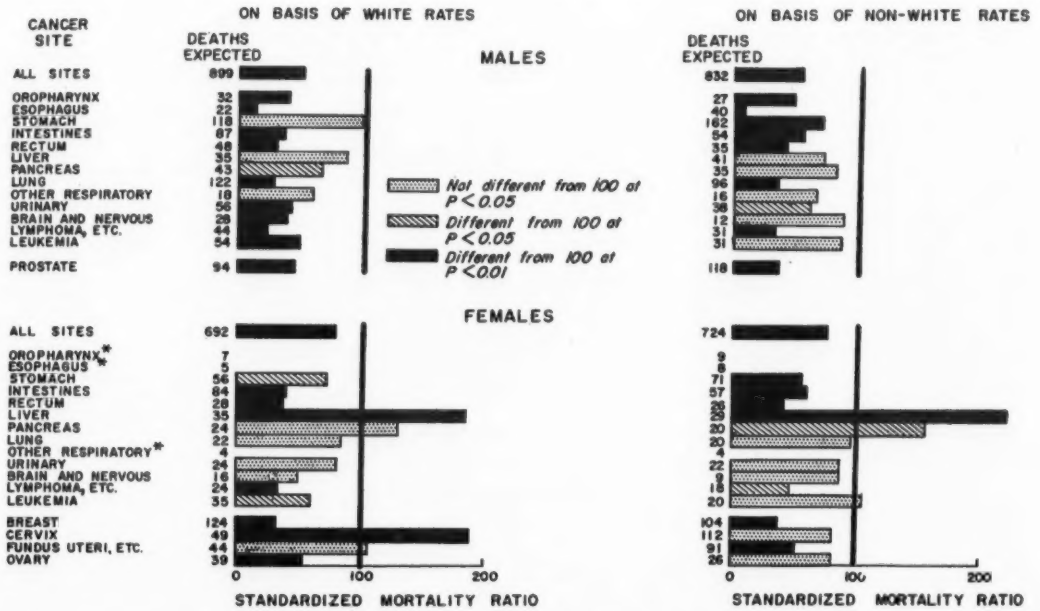
CANCER MORTALITY, INDIANS OF THE UNITED STATES, 1949 - 1952



TEXT-FIGURE 1.—Malignant neoplasms, selected sites: SMR's for Indians of the United States, 1949–52, and deaths expected among them on the basis of the United States white and United States nonwhite rates, 1950–51.

*SMR's not shown if both expected and recorded deaths are fewer than 9.

TABLE 2.—*Cancer deaths recorded among the Indians of the United States during 1949–52, inclusive, expected deaths for the same period on the basis of age-specific rates for 1950 and 1951 among United States white and nonwhite males and females, and resultant standardized mortality ratios (SMR's)*

Cancer site	International list number	Males					Females				
		Number of recorded deaths	Basis of U. S. nonwhite rates		Basis of U. S. white rates		Number of recorded deaths	Basis of U. S. nonwhite rates		Basis of U. S. white rates	
			Number expected deaths	SMR	Number expected deaths	SMR		Number expected deaths	SMR	Number expected deaths	SMR
Cancer all sites	*(140–205)*	*461*	*831. 8*	*55**	*899. 2*	*51**	*549*	*723. 7*	*76**	*692. 1*	*79**
Lip, tongue, oral cavity, and pharynx	*(140–148)*	*13*	*26. 6*	*49**	*32. 2*	*40**	*8*	*8. 7*	†	*6. 8*	†
Lip, tongue, and oral cavity	(140–144)	4	14. 3	28*	19. 9	20*	4	5. 5	†	4. 4	†
Pharynx	(145–148)	9	12. 3	73	12. 3	73	4	3. 2	†	2. 4	†
Digestive tract and peritoneum	*(150–156A, 157–159)*	*229*	*376. 2*	*61**	*359. 1*	*64**	*190*	*218. 7*	*87*	*237. 2*	*80**
Esophagus	(150)	3	39. 8	8*	22. 3	13*	6	8. 1	†	5. 0	†
Stomach	(151)	115	161. 9	71*	117. 8	98	40	70. 8	56*	55. 6	72‡
Intestines and rectum	*(152–154)*	*46*	*89. 2*	*52**	*135. 0*	*34**	*45*	*83. 3*	*54**	*112. 9*	*40**
Intestines	(152–153)	31	54. 3	57*	87. 3	36*	34	57. 1	60*	84. 4	40*
Rectum	(154)	15	34. 9	43*	47. 7	31*	11	26. 2	42*	28. 5	38*
Liver and biliary passages	*(155–156A)*	*30*	*41. 3*	*73*	*34. 6*	*87*	*64*	*28. 6*	*224**	*34. 6*	*185**
Liver and biliary passages, primary	(155)	12	15. 1	79	14. 6	82	29	10. 1	287*	18. 7	155‡
Liver, unstated whether primary or secondary	(156A)	18	26. 2	69	20. 0	90	35	18. 5	189*	15. 9	220*
Pancreas	(157)	29	35. 0	83	43. 4	67‡	31	19. 7	157‡	24. 1	129
Peritoneum, other and unspecified	(158–159)	6	9. 1	66	6. 0	†	4	8. 2	†	5. 0	†
Respiratory system	*(160–164)*	*45*	*112. 1*	*40**	*140. 9*	*32**	*22*	*23. 5*	*94*	*25. 9*	*85*
Trachea, bronchus, and lung	(162–164)	34	95. 6	36*	122. 4	28*	19	19. 6	97	22. 4	85
Other	(160, 161, 164)	11	16. 5	67	18. 4	60	3	3. 9	†	3. 5	†
Breast and genitourinary organs	*(170–181)*	*76*	*166. 2*	*46**	*160. 7*	*47**	*221*	*259. 8*	*61**	*283. 7*	*78**

Breast and genitourinary organs	(170–181)	*76*	*166. 2*	*46**	*160. 7*	*47**	*221*	*359. 8*	*61**	*283. 7*	*78**
Breast	(170)	1	2. 0	†	1. 8	†	40	103. 9	38*	123. 6	32*
Genital organs	(171–179)	*51*	*125. 8*	*40**	*103. 3*	*49**	*162*	*234. 1*	*69**	*136. 6*	*118‡*
Uterus	(171–174)	—	—	—	—	—	*139*	*202. 9*	*68**	*93. 4*	*149**
Cervix	(171)	—	—	—	—	—	92	112. 2	82	49. 0	188*
Other and unspecified parts of uterus	(172–174)	—	—	—	—	—	47	90. 7	52*	44. 4	106
Ovary	(175)	—	—	—	—	—	21	25. 8	81	38. 8	54*
Other and unspecified female genital organs	(176)	—	—	—	—	—	2	5. 4	†	4. 4	†
Prostate	(177)	43	118. 3	36*	94. 1	46*	—	—	—	—	—
Testis, other, and unspecified male	(178–179)	8	7. 5	†	9. 2	87	—	—	—	—	—
Kidney, urinary bladder, and other urinary	(180–181)	24	38. 4	62‡	55. 6	43*	19	21. 8	87	23. 5	81
Other and unspecified sites	(*156B, 165, 190–199*)	*61*	*88. 4*	*69**	*108. 1*	*56**	*79*	*75. 6*	*104*	*79. 7*	*99*
Skin	(190–191)	4	6. 0	†	17. 4	23*	5	5. 8	†	10. 1	50
Brain and other nervous system	(193)	11	12. 4	89	27. 9	39*	8	9. 1	88	16. 0	50
Bone and connective tissue	(196–197)	9	12. 3	73	14. 6	62	8	8. 5	†	8. 7	†
Other	(156B, 165, 192, 194, 195, 198–199)	37	57. 7	64*	48. 2	77	58	52. 2	111	44. 9	129
Leukemia and lymphoma	(*200–205*)	*37*	*62. 3*	*59**	*98. 2*	*38**	*29*	*37. 4*	*78*	*58. 8*	*49**
Lymphoma and mycosis fungoides	(*200–203, 205*)	*10*	*31. 3*	*32**	*44. 1*	*23**	*8*	*17. 5*	*46‡*	*23. 8*	*34**
Lymphosarcoma and reticulosarcoma	(200)	3	11. 8	25‡	19. 4	15*	—	6. 0	†	10. 6	0
Hodgkin's disease	(201)	1	9. 9	10*	15. 1	7*	3	5. 9	†	7. 7	†
Other lymphoma and mycosis fungoides	(202, 203, 205)	6	9. 6	62	10. 6	57	5	5. 6	†	5. 5	†
Leukemia and aleukemia	(204)	27	31. 0	87	54. 1	50*	21	19. 9	106	35. 0	60‡

*P = <0.01.

†No SMR computed where both recorded and expected deaths are fewer than 9.

‡P=0.01 to 0.05.

Italicized figures represent totals of 2 or more listed specific cancer sites.

the ratio for cancer of the fundus and other and unspecified parts of the uterus in Indians is significantly lower in comparison with nonwhites, while compared with whites no appreciable difference is seen. It is interesting to note that Indian females had only 5 recorded deaths due to malignancy of the skin compared to 10 expected if white female rates had prevailed, but the numbers are too small for the difference to be significant.

Discussion

The uncertainty as to the correct population base to use in computation of expected deaths makes it necessary to be cautious in placing any emphasis on proportionally small differences between observed and expected deaths. The estimate used appears to be a much closer approximation to the true base than the 1950 Census, which is only 80 percent of the estimate. Computations, not shown, were made based on the assumption of a population which was 90 percent of the estimate used, and they showed that the level of significance would be changed in very few instances—none of them of much importance in the over-all comparison. Thus, it is believed that reliance can be placed, from the statistical standpoint, on the observed differences reported, particularly those where proportionally large differences are shown.

Indians of the United States are geographically widely dispersed and the total population comprises numerous tribes and varied admixtures of blood from other races. Therefore, in addition to social and cultural differences between tribes, Indians cannot be considered a racially pure group. However, according to Birdsell (*9*) "... most anthropologists accept the presence of a Mongoloid genetic element as undeniable in all the populations of American Indians. The majority consider that they show evidence also of a contribution from some non-Mongoloid source, ..." which Birdsell believes to be Amurian, a variant of the Caucasoid race. Therefore, it seems logical to regard the American Indians as a racial group and to compare vital data recorded for them to that recorded for other races.

In evaluating comparisons of expected and observed causes of death discussed above, it must be borne in mind that an excessive number of deaths were attributed to senility and ill-defined causes (table 1). Many of these deaths in Indians 45 years of age and older, the age of greatest cancer risk, may have been due to malignant disease. Table 3 shows

TABLE 3.—*Deaths attributed to senility and ill-defined causes among United States Indians, 1949–52, inclusive*

Age	Males	Females	Total
Under 45.....	424	361	785
45–64.....	106	92	198
65 and over....	354	314	668
Total.....	884	767	1, 651

the number of deaths attributed to senility and ill-defined causes according to sex and age group. Deaths attributed to malignant disease, as well as to cardiovascular-renal disease, are shown in table 4, together with the expected deaths on the basis of white or nonwhite rates. Also shown are the differences between the recorded and expected deaths, according to age and sex.

In comparing tables 3 and 4 it is readily seen that if most of the deaths attributed to senility and ill-defined causes had actually been due to cancer they would have been sufficient to account for the deficit computed for persons 45 years of age and older. However, it seems evident that a large proportion of unreliably certified deaths would have been ascribed to other causes, such as cardiovascular-renal disease, if the true diagnosis were known. Thus, on examining the deficits in mortality computed for cardiovascular-renal disease among Indians 45 years of age and older, shown in table 4, it is readily observed that the deaths attributed to senility and ill-defined causes fall far short of making up these deficits in all comparisons. However, for females 45 to 64 years of age there is actually a slight excess of mortality compared to that expected on the basis of white female death rates, but the excess is canceled by the large deficit among those 65 years of age and older.

There is also a question as to whether some of the markedly excessive Indian mortality attributed to diseases of the respiratory and digestive systems (table 1) was actually due to cancer. Analysis of the data by age group showed that most of the excess mortality among Indians occurred before the 5th year of age. Only a slight excess among males 45 years of age and over was present, in comparison to white male rates, while there was a slight deficit compared to nonwhite male rates. Among females in the same age bracket there was an excess mortality in comparison to both white and nonwhite rates, which was somewhat larger than the deficit computed for cancer mortality.

The marked excess of mortality attributed to pulmonary tuberculosis among Indians also occurs largely before the age of 45 years. However, among Indians 45 years of age and older there is a moderate excess mortality for respiratory tuberculosis which, for males, is considerably less than the deficit of cancer mortality in this age group. Among Indian females of this age the deficit of cancer mortality is comparable in numbers to the excess computed for tuberculosis mortality. The excess mortality for tuberculosis in the older age groups could account for the deficit noted for cancer of the lung among Indian males.

It would appear that the probability of misdiagnosing tuberculosis as cancer of sites other than lung would not be great except for those unrecognized malignancies in which lung metastasis dominates the clinical picture. This would also be true in regard to other diseases of the respiratory system. Deaths due to conditions such as diseases of the cardiovascular-renal system could be misdiagnosed as a disease of the respiratory or digestive system. Thus, the marked deficit in mortality attributed to cardiovascular-renal disease among United States Indians, coupled with

TABLE 4.—*Deaths attributed to cancer and to cardiovascular-renal disease among United States Indians, 1949–52, inclusive, deaths expected among them on the basis of white or nonwhite death rates, and differences between recorded and expected deaths*

Age	Males					Females				
	Recorded deaths	Basis white rates		Basis nonwhite rates		Recorded deaths	Basis white rates		Basis nonwhite rates	
		Deaths expected	Difference	Deaths expected	Difference		Deaths expected	Difference	Deaths expected	Difference
Cancer										
Under 45	76	110	−34	104	−28	133	121	+12	163	−30
45–64	151	302	−151	368	−217	222	244	−22	319	−97
65 and over	234	487	−253	360	−126	194	327	−133	242	−48
Total	461	899	−438	832	−371	549	692	−143	724	−175
Cardiovascular-renal disease										
Under 45	247	194	+53	425	−178	232	108	+124	426	−194
45–64	628	991	−363	1, 573	−945	385	372	+13	1, 150	−765
65 and over	1, 274	2, 369	−1, 095	2, 229	−955	914	1, 663	−749	1, 730	−816
Total	2, 149	3, 554	−1, 405	4, 227	−2, 078	1, 531	2, 143	−612	3, 306	−1, 775

the observed low cancer mortality, strengthens the hypothesis that the deficit in deaths ascribed to malignant disease among Indians is real.

Conclusion

Analysis of mortality recorded among the Indians of the United States supports the view that they show a deficit of cancer as a whole. Deficits of varied degree were observed among males for all sites. For females, lower cancer mortality was demonstrated for most of the sites, while excesses were shown for mortality attributed to cancer of the liver, and possibly the pancreas, compared to white and nonwhite rates, and for cancer of the cervix, compared to white rates. Sites showing marked deficits were intestines and rectum and lymphomas for both sexes. For males, marked deficits were observed for oral cavity, esophagus, lung, urinary system, and prostate, compared to both white and nonwhite rates. Cancer of the skin, brain, and nervous system and mortality ascribed to leukemia were less frequent among Indian males than among whites. Among Indian females mortality attributed to cancer of the breast was markedly deficient compared to both white and nonwhite rates, while compared to nonwhite rates there was a deficit attributed to malignant disease of the uterus other than cervix.

These demonstrated deficits in mortality should not be unreservedly accepted as true deficits because analyses by broad causes have shown an excess of deaths attributed to senility and ill-defined causes among Indians 45 years of age and older. If most of these had actually been due to cancer, they would have more than accounted for the observed deficit of cancer mortality in the corresponding age groups. In addition, there is an excess mortality among Indians aged 45 years and older attributed to diseases of the digestive tract and respiratory system, including tuberculosis. The excess among Indian men is not enough to account for the observed deficit of cancer deaths but could account for the deficit for cancer of the lung. The excess among Indian women is somewhat larger than the computed deficit of cancer deaths. However, the deaths unreliably certified as to cause and the excess mortality for diseases of the digestive tract and respiratory system are not nearly enough to account for the deficit computed for deaths attributed to cardiovascular-renal disease. Thus, it seems quite likely that the low cancer-death rate observed among American Indians is real.

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Mortality Attributed to Cancer among Hawaiians and Filipinos of Hawaii and Other Racial Groups of the United States and Hawaii [1]

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There is evidence from a few published statistical analyses of clinical and autopsy material suggesting that Filipinos and Hawaiians are subject to risks of cancer of several sites that differ from those observed in the white race. Steiner (*1*) noted that there is only scattered information on the frequency of cancer among Filipinos in the Philippine Islands. Those data that are available are not in close agreement regarding the relative frequency for cancer of different sites. However, it appears that there is a high proportion of cancer of the liver (*2–4*) and uterus (*2–6*) in relation to all cancer in the Philippines. For Hawaii, Quisenberry (*7*) presents morbidity data indicating that cancer of the liver is relatively more frequent among Filipinos and Hawaiians than among Caucasians. He also reports (*8*) that mortality attributed to all cancer, cancer of the stomach, and cancer of the female breast is considerably higher among Hawaiians than among Caucasians of Hawaii. Hoffman (*9*) in an earlier study presented data showing that mortality attributed to all cancer was very high among full-blooded Hawaiians. Steiner (*1*) found that in Los Angeles the relative frequency of cancer of the liver was probably greater among Filipinos than among Caucasoids. Thus, in view of the above observations, this study was initiated to compare cancer mortality of minor racial groups, including Hawaiians and Filipinos of Hawaii, as well as a mixed racial group in the United States composed largely of Filipinos, to cancer mortality among the white and nonwhite populations of the United States.

Method of Study

Special tabulations of deaths attributed to malignancy during 1949 to 1952, inclusive, among minor racial groups of the United States and Hawaii were obtained from the National Office of Vital Statistics. For Hawaii the data were given specifically for Hawaiians and part-Hawaiians, Filipinos, Caucasians, Chinese, Japanese, and other races. For the United States the tabulations included Japanese, Chinese, Indians, and other races. Detailed analyses of the data relating to the Japanese and Chinese

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of both areas, as well as to American Indians, have already been made by the author (*10–12*). Comparisons are made in this study between mortality of the remaining racial groups and the white and nonwhite death rates.

Deaths recorded in each minor racial group were compared to deaths expected among them during the same 4-year period had they been subject to the age-, sex-, and site-specific death rates of the white or nonwhite populations of the United States for 1950 and 1951 (*13*). The observed deaths divided by those expected yields a ratio. This ratio, multiplied by 100, gives a standardized mortality ratio (SMR) which numerically represents the percentage that the age adjusted average annual rate of the minor racial group under consideration is of the United States death rate.

Results

Because of the relatively small populations involved there were too few deaths observed or expected in many of the sites to allow a comparison of mortality rates. Therefore, in presenting the data, only those sites are tabulated in which there were sufficient numbers in one or more of the comparisons. These results are shown separately by race and sex in table 1.

Hawaiians

The Hawaiians in this study included persons who were partly of Hawaiian blood, as well as full-blooded Hawaiians, and comprised an estimated population of 87,723 as of January 1, 1951.

From table 1 it is readily seen that Hawaiian males have a higher death rate for all cancer than either white or nonwhite males. No significant differences are noted in similar comparisons of cancer mortality among Hawaiian females, although a few more deaths were recorded for them than expected on the basis of white female rates. More deaths were recorded than expected among Hawaiian males for cancer of the stomach, liver, and respiratory system, principally lung. The difference between observed and expected deaths for cancer of the stomach in the comparison with male nonwhite rates was too small to be significant. Among Hawaiian females there was also a marked excess of mortality attributed to cancer of the stomach and an excess for malignancies of the respiratory system, although the figures for the latter were too few for computation of reliable SMR's. There were fewer deaths recorded among females for cancer of the intestines and rectum than expected on the basis of either white or nonwhite rates, but the difference is significant only in the comparison with white rates. There were significantly more deaths attributed to lymphosarcoma and leukemia among Hawaiian males than expected on the basis of nonwhite mortality. Deaths attributed to cancer of the breast among Hawaiian females were slightly fewer, though not significantly, than expected on the basis of either white or nonwhite rates. On the other hand, deaths recorded as due to cancer of the uterus were significantly more than expected on the basis of white rates and somewhat fewer than expected if nonwhite rates had prevailed. Deaths due to cancer of the prostate are somewhat fewer than expected in both the nonwhite and

white comparisons but the deficiency was not as marked as among Japanese (*10*), Chinese (*11*), United States Indians (*12*), and the other minor racial groups, compared in the following sections.

Filipinos of Hawaii

The sex and age distribution of the Hawaiian Filipino population was remarkably uneven, according to the 1950 Census (*14*). Males 35 years of age and over comprised 60 percent of the total male population, while females of this age bracket comprised only 20 percent of the total female population. Since only 30 percent of the Filipino population, estimated at 61,699 as of January 1, 1951, was female, relatively few cancer deaths were recorded or expected for this sex.

Table 1 reveals that Filipinos of both sexes had significantly lower cancer mortality than expected on the basis of either white or nonwhite death rates. In contrast to Hawaiian males the Filipinos had fewer reported deaths attributed to cancer of the stomach and respiratory system than expected on the basis of either white or nonwhite male mortality. These differences were significant, except for cancer of the stomach, when compared to white rates. For Filipino females, on the other hand, although the numbers are small, it is interesting to note that 4 cases of cancer of the respiratory system were recorded, whereas only 1 would be expected on the basis of white or nonwhite rates, while no malignancies of the stomach were recorded among female Filipinos. A significant excess of cancer deaths was demonstrated for only 1 site, namely, cancer of the liver among Filipino males. Mortality due to malignancies of the intestines and rectum, pancreas, urinary system, and to the lymphomas and leukemias was somewhat lower among Filipino males in comparison to whites or nonwhites.

There is a large proportionate deficit of deaths attributed to cancer of the prostate among Filipino males in comparison with both white and nonwhite rates, though the numbers are too small, in the comparison with white male mortality, for computation of a meaningful SMR. Attention is called to the fact that only 1 cancer of the breast was recorded among Filipino women compared to 5 expected on the basis of either the white or nonwhite figures, while cancer deaths attributable to cancer of the uterus were almost as expected on the basis of white rates and fewer than expected if nonwhite rates had prevailed.

Other Races of Hawaii

Since the group designated as "other races" of Hawaii, estimated at 21,215 as of January 1, 1951, comprises a number of groups of different national origins and racial stocks, including Koreans, Puerto Ricans, Negroes, and others, no detailed discussion is justifiable. In passing, it is worthwhile to note (table 1) that among the males of this group, mortality attributed to cancer of the stomach and the respiratory system is greater than expected on the basis of white or nonwhite rates, while there is a deficit in mortality attributed to cancer of the prostate.

TABLE 1.—*Cancer deaths recorded among Hawaiians and Filipinos of Hawaii and among other racial groups of Hawaii and the United States during 1949–52, inclusive, deaths expected for the same period on the basis of age-specific rates for 1950 and 1951 among the United States white and nonwhite males and females, and resultant standardized mortality ratios (SMR's)*

Selected site	International list number (6th revision)	Males					Females				
		Number deaths recorded	On basis of U. S. nonwhite rates		On basis of U. S. white rates		Number deaths recorded	On basis of U. S. nonwhite rates		On basis of U. S. white rates	
			Number deaths expected	SMR	Number deaths expected	SMR		Number deaths expected	SMR	Number deaths expected	SMR
Hawaiians											
All cancer	*(140–205)*	*124*	*99. 4*	*125**	*94. 3*	*131†*	*110*	*112. 4*	*98*	*95. 9*	*115*
Stomach	(151)	25	17. 1	146	10. 6	236†	28	9. 4	298†	6. 2	452†
Intestines and rectum	(152–154)	9	9. 4	96	12. 7	71	6	11. 6	52	13. 5	44*
Liver	(155–156A)	11	4. 7	234†	3. 3	333†	2	4. 1	‡	4. 0	‡
Respiratory system	(160–164)	29	13. 5	215†	15. 7	185†	8	3. 5	‡	3. 4	‡
Breast	(170)	—	0. 2	‡	0. 2	‡	13	16. 3	80	17. 9	73
Uterus	(171–174)	—	—	—	—	—	23	33. 1	69	14. 0	164*
Prostate	(177)	5	10. 1	50	6. 6	‡	—	—	—	—	—
Lymphoma and leukemia	(200–205)	18	9. 7	186*	14. 5	124	5	7. 1	‡	10. 4	48
Filipinos (Hawaii)											
All cancer	*(140–205)*	*111*	*192. 7*	*58†*	*166. 3*	*67†*	*18*	*36. 6*	*49†*	*29. 3*	*61**
Oropharynx	(140–148)	6	7. 6	‡	6. 0	‡	—	0. 4	‡	0. 2	‡
Esophagus	(150)	3	11. 1	27*	4. 0	‡	1	0. 4	‡	0. 1	‡
Stomach	(151)	11	36. 8	30†	19. 1	58	—	2. 8	‡	1. 5	‡
Intestines and rectum	(152–154)	13	19. 2	68	23. 1	56*	2	3. 5	‡	3. 6	‡
Liver	(155–156A)	22	9. 8	224†	6. 2	355†	—	1. 3	‡	1. 1	‡
Pancreas	(157)	2	8. 8	‡	8. 5	‡	—	0. 9	‡	0. 7	‡
Respiratory system	(160–164)	17	32. 7	52†	35. 4	48†	4	1. 1	‡	1. 0	‡
Breast	(170)	—	0. 6	‡	0. 4	‡	1	5. 3	‡	5. 6	‡
Uterus	(171–174)	—	—	—	—	—	4	10. 9	37	4. 4	‡
Prostate	(177)	2	16. 7	12†	7. 7	‡	—	—	—	—	—
Urinary	(180–181)	3	8. 7	‡	9. 7	31*	—	1. 1	‡	0. 8	‡
Lymphoma and leukemia	(200–205)	11	15. 6	71	20. 5	54*	2	2. 6	‡	3. 8	‡

Other Races§ of Hawaii

All cancer	*(140–205)*	*75*	*56.6*	*133**	*67.2*	*112*	*20*	*28.2*	*71*	*25.3*	*79*
Stomach	(151)	26	11.4	228†	9.5	274†	—	2.5	‡	1.8	‡
Intestines and rectum	(152–154)	5	6.5	‡	10.7	47	1	3.1	‡	3.8	‡
Liver	(155–156A)	3	2.8	‡	2.6	‡	1	1.1	‡	1.2	‡
Respiratory system	(160–164)	13	6.6	197*	9.2	141	1	0.9	‡	0.9	‡
Breast	(170)	—	0.1	‡	0.1	‡	2	4.1	‡	4.7	‡
Uterus	(171–174)	—	—	—	—	—	4	8.2	‡	3.6	‡
Prostate	(177)	1	9.9	10†	8.7	‡	—	—	—	—	—
Lymphoma and leukemia	(200–205)	5	3.7	‡	6.2	‡	4	1.6	‡	2.4	‡

Other Races‖ of United States

All cancer	*(140–205)*	*151*	*296.8*	*51†*	*266.4*	*57†*	*27*	*89.0*	*30†*	*76.1*	*35†*
Oropharynx	(140–148)	7	11.3	62	9.5	74	—	1.0	‡	0.7	‡
Esophagus	(150)	4	16.6	24†	6.3	‡	—	1.0	‡	0.4	‡
Stomach	(151)	20	56.6	35†	31.3	64	4	7.3	‡	4.9	‡
Intestines and rectum	(152–154)	19	29.9	64	37.4	51†	—	9.1	0†	10.6	0†
Liver	(155–156A)	16	15.0	107	9.9	162	1	3.2	‡	3.2	‡
Pancreas	(157)	3	13.4	22†	13.4	22†	1	2.1	‡	2.2	‡
Respiratory system	(160–164)	27	48.6	56†	53.3	51†	1	2.8	‡	2.7	‡
Breast	(170)	1	0.9	‡	0.6	‡	—	12.9	0†	14.1	0†
Uterus	(171–174)	—	—	—	—	—	5	26.4	19†	11.1	45
Prostate	(177)	1	28.0	4†	15.3	6†	—	—	—	—	—
Urinary	(180–181)	7	13.6	51	15.6	45*	1	2.6	‡	2.3	‡
Lymphoma and leukemia	(200–205)	17	24.1	71	32.8	52†	6	5.8	‡	8.5	‡

* Difference significant at $P = 0.01$ to 0.05.

† Difference significant at $P < 0.01$.

‡ No SMR computed if both expected and recorded deaths are fewer than 9.

§ Races other than Caucasian, Hawaiian Chinese, Japanese, and Filipino.

‖ Races other than White, Negro, Japanese, Chinese, and Indian.

Other Races of the United States

The population comprising the "other-races" group of the United States was enumerated as 110,004 in April 1950, of which Filipinos make up 56 percent. Hadley (*15*) states that the 1950 Census enumerated as "other races" an estimated 30,000 persons classed as "Indians" in previous censuses. He presents evidence indicating that vital events in a sizable but unknown proportion of these 30,000 people are reported to the National Office of Vital Statistics as occurring among "Indians." Thus the population base used to compute expected deaths in the "other races" of the United States is greater than the true base. Therefore, it must be borne in mind that the expected deaths are somewhat more than they actually should be and the resultant SMR's are, consequently, lower than the true value for any given site.

The age and sex distribution of the "other-races" group resembles that of the Filipinos of Hawaii. Since Filipinos comprise such a large segment of this group and since two thirds of the males are Filipinos, one would expect the results of the analysis to be similar to that for cancer mortality among Filipinos of Hawaii, especially among males.

On comparing the tabulations in table 1 a close resemblance can be seen. The only difference of much importance is that there is a marked excess of mortality attributed to cancer of the liver in the Filipino males of Hawaii compared to the deaths expected, while an excess is present among the "other-races" group of the United States, but the difference is proportionally smaller and not statistically significant. There are other minor differences. No excess mortality from cancer of the respiratory system was noted among females of the "other-races" group, in contrast to the situation noted for female Filipinos of Hawaii. As was the case for Filipino females of Hawaii, mortality attributed to intestines and rectum, breast, and uterus is lower among the females of the "other-races" group of the United States than expected on the basis of white or nonwhite rates. In contrast to the Filipino females of Hawaii, however, all these mortality deficits are significant, except for uterus, in comparison to white female rates. Unfortunately, data were not readily available to check specifically on deaths reported among Filipinos of the United States, but it would appear from the above analysis that an even closer resemblance would be demonstrated were the data at hand for analysis.

Discussion

Due to the relatively small populations of the racial groups under consideration, cancer mortality among them could be adequately analyzed only for certain selected sites for which sufficient data were available to permit comparison of recorded or expected deaths. Furthermore, for the same reason, the only adequate method of comparison with white or nonwhite rates was by the indirect method of age adjustment. An SMR computed by the indirect method of age adjustment should not be compared to the SMR of another race for the same site, especially when considerable differences exist between the age distributions of the 2 popula-

tions. However, SMR's computed by the direct method of age adjustment for all cancer and for a few sites with fairly substantial numbers of deaths agreed quite closely with those computed by the indirect method in each racial group. In addition, comparison of age- and sex-specific death rates also substantiated observed differences in mortality. Therefore, it would appear, in view of the wide variations in values of SMR's for several specific sites among the racial groups, that general conclusions may be drawn as to wide differences or close similarities of cancer mortality observed in this presentation.

Thus, there appears to be close similarity in cancer mortality between the Filipinos of Hawaii and the "other-races" group of the United States, which is largely Filipino in composition. The only major difference is that mortality attributed to cancer of the liver was markedly higher among Filipino males of Hawaii than expected on the basis of either white or nonwhite rates but was only moderately higher among the "other races" of the United States than expected.

The observation of other investigators discussed above regarding the high relative frequency of cancer of the liver among Filipinos is in line with the excess mortality among Filipino males of Hawaii found in this study. On the other hand, since a high relative frequency of deaths due to any cause does not necessarily reflect an unusually high mortality rate, it is possible that the high proportion of uterine cancer previously noted among women of the Philippines does not imply a high death rate for cancer of the uterus among them. The analysis presented here did not show that mortality attributed to cancer of the uterus was excessive among the Filipino women of Hawaii. In fact, there were actually fewer deaths ascribed to cancer of the uterus than expected among them on the basis of nonwhite female rates. Too few deaths were involved to attach much importance to this finding, however.

The Hawaiians represent all gradations of admixture of blood of various races and cannot be considered a racially pure group. Nevertheless, the marked excess of mortality attributed to cancer of the stomach, liver, and respiratory system are interesting findings and seem deserving of further study. Some of the findings reported here have been suggested previously in relative-frequency comparisons, as indicated above. However, this study did not confirm the findings of Quisenberry (*7,8*) that mortality attributed to cancer of the breast is high among Hawaiians, possibly because his data represent an earlier period than data presented above. The morbidity data presented by Quisenberry do show, however, that breast cancer is less frequently diagnosed among Hawaiian than among Caucasian females of Hawaii. An interesting observation is the fact that the Hawaiians have fewer deaths due to cancer of the prostate than expected on the basis of either white or nonwhite death rates. The observed deficit among Hawaiians, however, does not approach the magnitude of the deficit demonstrated in the Filipinos of Hawaii, residual racial groups of the United States, Japanese (*10*), Chinese (*11*), and American Indians (*12*).

Computations not shown here revealed that cancer mortality among Caucasians of Hawaii did not differ significantly from the mortality expected among them for any site on the basis of the death rates of the white population of the United States.

Summary

Mortality recorded during 1949–52 among Hawaiians and Filipinos of Hawaii and a population group in the United States, most of whom were Filipinos, is compared to deaths expected among them if they had been subject to the death rates of the white or nonwhite populations of the United States.

As would be expected, the standardized mortality ratios computed for Filipinos of Hawaii and the residual racial group of the United States were comparable for most sites. The main difference was a marked excess of recorded mortality for cancer of the liver among the male Filipinos of Hawaii, compared to a proportionally smaller excess in the group residing in the United States. Filipinos had marked deficits in total cancer mortality for both sexes. Among males there were deficits for cancer of the stomach, intestines and rectum, pancreas, respiratory system, and prostate. Among Filipino females a deficit was shown for deaths attributed to cancer of the breast, though the numbers were too small to be considered of much significance. An interesting observation is the fact that mortality attributed to cancer of the uterus was not excessive among Filipinos but was actually less than expected on the basis of death rates of the nonwhite females of the United States.

Hawaiian males were shown to have significantly higher mortality for all cancer than the white or nonwhite populations of the United States. This excess mortality was attributable chiefly to deaths ascribed to cancer of the stomach, liver, and respiratory system among Hawaiian males. Hawaiian females had many more deaths attributed to cancer of the stomach than expected, while mortality ascribed to cancer of the uterus was greater than expected on the basis of white rates and somewhat less than had nonwhite female rates prevailed.

The Caucasians of Hawaii showed no significant differences between observed deaths and deaths expected for any site on the basis of cancer mortality of the white population of the United States.

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Relationship Between Chances of Survival and Regeneration of Hematopoietic Tissue in Irradiated Rats [1, 2]

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Previous publications from this laboratory (*1–4*) have demonstrated that animals X-irradiated with an LD100/30 and protected against death by shielding parts of the body or by injecting bone marrow or 2-mercaptoethylamine had the same injuries to bone marrow and spleen, histologically, as unprotected controls given the same dose of radiation. An exception must, of course, be made for those organs shielded during exposure to X irradiation, where the shielding considerably reduced the damage (*1–3*).

Bloom also (*5*) pointed out that he could see no difference in the histologic damage observed in animals that died after an LD50/30 and those that did not die after the same dose. De Bruyn (*6*), in a systematic study of the problem, examined lymphoid tissue of rats, guinea pigs, and rabbits that had received local and total-body doses of X radiation and found numerous discrepancies between final survival and degree of injury. He concluded that the degree of visible injury expressed the dose received rather than the chances of survival. These observations hold true when animals are protected or treated against an LD100/30.

When an attempt is made to prevent death from X irradiation, some animals die spontaneously and others survive long enough to be sacrificed for histologic purposes. It has been demonstrated (*1–4*) that there was no difference in the degree of histologic injury between animals that died spontaneously and those that were sacrificed. A clear-cut difference between these groups was observed in the regeneration of the bone marrow and spleen. While extensive regeneration was found in many of the sacrificed animals, it was absent in those dying spontaneously. These experiments also showed that the most effective methods of preventing death induced earlier regeneration of bone marrow and spleen in the irradiated animals. However, the number of animals observed in each group was too small to correlate the frequency of regeneration in the protected animals with the frequency of survival.

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[3] With the technical help of Miss H. Hoeck, to whom the author wishes to express his gratitude.

A study of this correlation was the aim of the present experiment. In order to be conclusive, it is evident that an experiment correlating histologic data and survival curves must provide optimal opportunity for the animals that are sacrificed for histologic observation to behave in the same manner as those used to establish the mortality curve. Therefore, a large number of animals was used and a method of preventing death that would provide reasonably reproducible results. Theoretically, the same animals used for establishing the survival curve should also be used for carrying out the histologic observations. This, of course, is impossible, but it may be achieved to some extent by using an artifice which provides adequate survival curves.

Methods

Correlated observations were carried out on a group of 67 animals irradiated with 700 r, with the hepatic zone shielded as described before (*3*). This method of preventing death was chosen because it was established, from 6 different mortality curves on groups of 24 rats, that the general shape of the curves was sufficiently reproducible. The final mortality, as shown by these curves, was 55 ± 4 percent.

The spontaneous-mortality rate of the irradiated animals was expressed in the following way: The number of surviving animals (A) was counted each day by subtracting both the number of sacrificed animals (B) and the number of animals dying as the result of irradiation (C). Each morning the number of animals that had died in the preceding 24 hours from the effects of irradiation was expressed as a percentage of the corrected number of survivors calculated on the previous morning (D). Suppose that on the 3rd day $A-(B+C)=D$. If the number of spontaneously dying animals is X on the 4th day, the percentage (Y) will be calculated in the following way: $Y=\frac{X}{D}\times 100$. This value gives the rate of death for each day. By adding the daily rates of death, a curve of cumulative frequencies is constructed expressing the percentage of all the animals surviving on each day in proportion to the initial value of 100 percent.

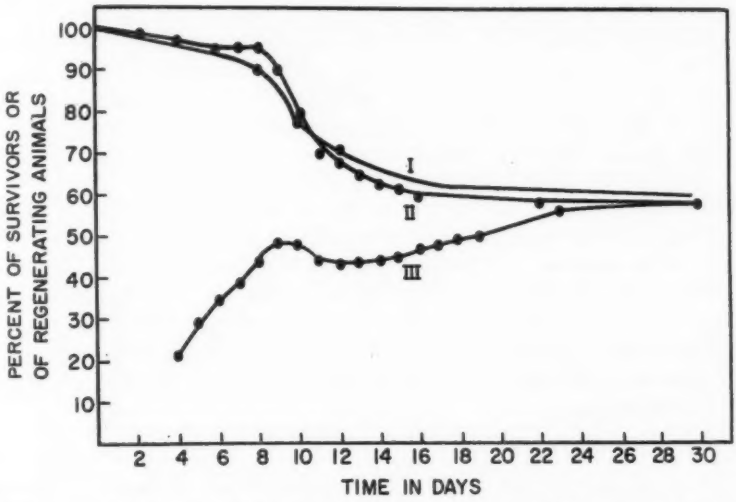
As in the former experiments (*1–4*), rats of the Wistar strain were used. The average weight of the animals was 150 ± 5 gm. The conditions of irradiation and the histologic techniques were also identical to those previously described (*3–4*).

The histologic results were compared with those in 10 sacrificed controls that received 700 r without shielding of the hepatic zone. The spleens were weighed on a Metler balance at the time of autopsy.

Results

As demonstrated (text-fig. 1), each point of the survival curve plotted as described can be superimposed with ±3 percent accuracy on the corresponding points of a standard curve established with 106 rats weighing 150 gm. and receiving 700 r, with the hepatic zone shielded (curve I).

It is thus quite reasonable to assume that this group of animals, from which histologic samples were taken, responded to irradiation in the same manner as other groups used for study of the survival rate. Of the 67 animals, 14 were still alive on the 15th day, 11 had died spontaneously, and 42 had been killed on various days between the 1st and 15th days after irradiation. The 14 surviving animals were all killed between the 15th and 30th days after irradiation.



TEXT-FIGURE 1.—Curve I is the standard survival curve obtained on 106 animals, divided into groups of 24, irradiated with 700 r with the hepatic zone shielded during exposure. Curve II is the mortality curve obtained as described (*see* text). Curve III is the curve which expresses the frequency of regeneration as described (*see* text).

The histologic findings confirmed what has been described before (*3*). On examination of the histologic preparation of bone marrow, nearly complete depopulation of bone marrow was observed in all animals after the 4th day following irradiation until the time when regeneration started. On the 5th day, foci of undifferentiated cells could occasionally be found near the cartilage in the bone sections of the shielded animals dying spontaneously, as well as in the bone sections of 10 sacrificed controls that were irradiated without any shielding. The extent of regeneration never increased.

It therefore appeared that only the experimental animals with numerous foci (15 to 20) of immature and mature cells of the erythropoietic and myelopoietic series, disseminated throughout the entire section of the bone, could be considered as regenerating efficiently.

The sections of the spleen of the animals that died spontaneously and of the irradiated but unshielded controls also presented occasional foci of undifferentiated cells. Only the animals presenting numerous foci of

immature or mature cells of the lymphopoietic series, disseminated throughout the entire section of the spleen, were considered as regenerating efficiently.

The total number of animals showing regeneration of bone marrow was counted each day and was expressed as a percentage of the total number of animals dead at that time. These values were plotted in curve II (text-fig. 1). The area delimited by the 2 complementary curves (II and III) may be divided into 3 phases: 1) a zone ranging from the 4th to the 8th day when very few animals died but a large percentage (44 percent) of the examined animals had regenerated bone marrow; 2) a zone ranging from the 8th to the 14th day, with a considerable drop in the survival rate coinciding with reduction and momentary stabilization of the percentage of examined animals showing regeneration; 3) a zone ranging from the 14th to the 30th day when the survival curve is stabilized while the percentage of regenerating animals is still increasing.

A similar curve has been observed for the regeneration of the spleen, except that by the 8th day after irradiation 1 animal that had regenerated its bone marrow had not regenerated its spleen. On the 12th day an animal regenerated its spleen, while no significant regeneration of the bone marrow was observed. Fifty percent of the regenerating animals presented focal myelopoietic metaplasia between the 5th and 9th days, while all those examined between the 9th and 17th days showed numerous granulopoietic foci.

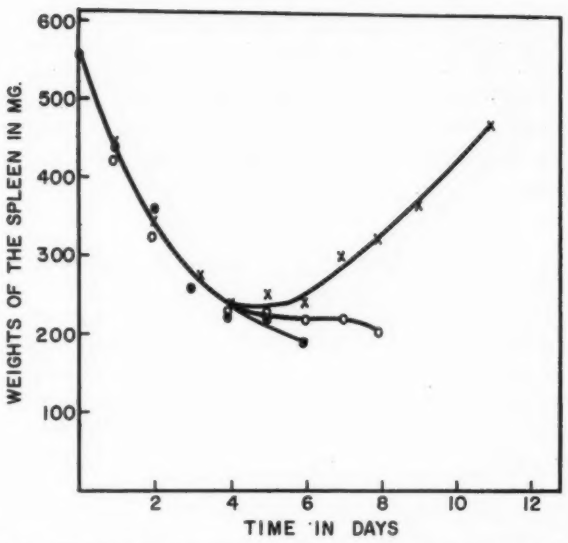
Spleen Weights

The values obtained for the animals that died spontaneously were plotted separately from those of the sacrificed animals, and both curves were compared with data obtained from the control group (text-fig. 2). The same drop in weight is observed in the 3 groups between the 1st and the 4th day. Later the curve of the animals that died spontaneously forms a plateau at these low values, while the values observed for the irradiated controls drop somewhat, but not significantly. The weights of the sacrificed animals increase slowly at first and rapidly later on.

Discussion

This experiment correlating survival and frequency of regeneration confirms the assumption that the presence of marked injury of bone marrow and spleen does not necessarily imply death of the animal, since all the animals sacrificed presented marked injury between the 1st and 4th days. On the other hand, 44 percent of all animals sacrificed between the 4th and 8th days of observation showed definite signs of regeneration of bone marrow. At the end of 30 days, all the animals examined had regenerated bone marrow and spleen.

A drop in the frequency of regeneration that coincides with the maximum death rate is also of interest. This drop was first described by Tsang (7), who studied the hematopoietic tissues of rabbits X-irradiated with total-body doses. Moreover, from his personal studies, Bloom (5) pre-



TEXT-FIGURE 2.—Weight of spleen on various days following exposure to X irradiation. Each point represents the mean value of data obtained from at least 5 animals. X = data for animals irradiated with 700 r with the hepatic zone shielded that were sacrificed; ● = data for the animals that died spontaneously; ○ = data for the controls that were irradiated without shielding.

dicted such a drop. Thus, it does not seem unreasonable to assume that a correlation exists between the chance of survival and the regeneration of bone marrow and spleen.

If one assumes, however, that early regeneration is an essential condition of survival, then only 44 percent of the irradiated animals would survive, whereas it has been shown from the study of several curves that the survival rate is 55 ± 4 percent. Forty-four percent is significantly below these values, so that at least some of the surviving animals presented a delayed regeneration of bone marrow and spleen. This experiment thus establishes the fact that all surviving animals regenerate, but it does not demonstrate the absolute necessity of early regeneration, although most of the animals which regenerated did so early. It is not at all evident that the regeneration of bone marrow and spleen is the cause of survival; this might be only the first morphologic manifestation of survival in an animal that is actually surviving for some reason as yet unknown.

If the data obtained by weighing the spleens are correlated with the histologic findings, it appears that only the first and the latter parts of the curve express a true histologic picture; that is, pure histologic damage from the 1st to the 4th days and, essentially, signs of regeneration between the 8th and 14th days. Between these 2 periods the weight curve is inadequate for dissociating injury from regeneration for 2 reasons: 1) The weight plotted is the mean value of a relatively small number of animals, of which only a fraction will regenerate their spleens; and the amount of

this fraction will vary with the method used to prevent death. 2) In the group of regenerating animals at that time there is never a pure histologic picture of regeneration alone but a mixture of injury and repair. To study a dose-reduction effect of a method of preventing death from X irradiation, the spleens should be weighed at a time when the histology demonstrates maximum damage without regeneration.

Summary

The correlation of the chance of survival and the degree of regeneration of bone marrow and spleen was studied in a group of 67 rats that received 700 r after the hepatic zone was shielded. It appears from this study that a chronologic correlation exists between the frequency of regeneration and the survival curve. The animals that died spontaneously did not regenerate bone marrow and spleen, but 44 percent of the sacrificed animals regenerated hematopoietic tissue early (before the 8th day). After 30 days all the animals that survived had regenerated bone marrow and spleen.

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Studies of a Plasma-Cell Neoplasm of the Mouse. I. Characterization of Neoplasm 70429, Including Its Sensitivity to Various Antimetabolites with the Rapid Development of Resistance to Azaserine, DON, and *N*-Methylformamide[1]

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In 1954, Dunn (*1*) described a transplantable plasma-cell neoplasm in a strain C3H mouse. This neoplasm has been designated 70429. Spontaneous plasma-cell tumors of the mouse occur only rarely (*2–4*) and, to date, no simple means of inducing these neoplasms is known. Neoplasm 70429 was the second recorded transplantable plasma-cell neoplasm of the mouse; Bichel (*5*) described another in 1951. When the following experiments were begun, neoplasm 70429 had been transplanted subcutaneously through 20 generations. This tumor was readily converted to ascitic form (*6*) on intraperitoneal passage and was used as such in the experiments reported here.

Metabolic antagonists, which possess antagonistic action in the biochemical pathways of purine-ring synthesis, have been shown to be growth inhibitors of certain lymphocytic leukemias of the mouse (*7–10*). To date the most extensively studied compounds have been the folic acid and purine analogues. In 1954, azaserine (O-diazoacetyl-L-serine) (*11*) and in 1956 DON (6-diazo-5-oxo-L-norleucine) (*12*) were described as antibiotics (*13,14*) and inhibitors of the growth of sarcoma 180 (*11,12*). Further characterization of their action has been described in reference to purine-ring synthesis in the pigeon-liver enzyme system. Here, these glutamine antagonists block the conversion of (α-*N*-formyl)glycinamide ribotide (FGAR) to (α-*N*-formyl)glycinamidine ribotide (FGAM), presumably by uniting more competitively with the necessary enzyme than the normal metabolite, glutamine (*15–17*). The present report extends the study of inhibition of neoplastic-cell growth by azaserine and DON. The response of the plasma-cell neoplasm 70429 to amethopterin, 6-mercaptopurine, and *N*-methylformamide (NMF) is included.

It has been demonstrated that certain lymphocytic neoplasms of the mouse develop resistance to antimetabolites; there are descriptions in the literature of resistance to folic acid and purine analogues. This subject

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has been reviewed recently (*18*). The development of resistance to these compounds has been a gradual process, involving the passage of the tumor, usually through 6 or more transplant generations. The resulting resistant tumors were stable genetically and maintained their resistance in the absence of the specific antimetabolite used to select them (*19*).

The development of resistance to antifolic and antipurine compounds, among several lymphocytic neoplasms, appeared to resemble the pattern of resistance to penicillin in microorganisms (*20*).

Resistance to the glutamine antagonist azaserine in the plasma-cell neoplasm 70429 appears to resemble more closely the streptomycin pattern of resistance in microorganisms (*20*). The development of certain resistant lines of this plasma-cell neoplasm through various methods of antimetabolite exposure and the characterization of these lines are described.

Materials and Methods

Origin and maintenance of the tumor.—Neoplasm 70429 arose in a 14-month-old strain C3H/He mouse in 1952. This mouse had been injected with methylcholanthrene and tricaprylin at 15 days of age. The tumor appeared as a large growth in the ileocecal region and, since these neoplasms most often originate at this site, was suspected to be of plasma-cell origin. Microscopic study confirmed this impression. The tumor was then maintained by subcutaneous passage (20 transfers) in C3H/He mice over a period of 3 years. At this time, it was converted to an ascitic tumor in our laboratory in 1 transfer generation and has since been maintained by intraperitoneal ascitic passage (fig. 1). A more detailed morphologic description of this neoplasm is to be given by Dr. Thelma B. Dunn, of the Laboratory of Pathology of this Institute (*21*). This paper deals only with the ascitic transplant generations of the tumor. Thus, generation 3 is in reality generation 23 (20 subcutaneous passages plus 3 ascitic passages). This tumor produces 1 to 4 ml. of nonbloody ascites in 7 to 14 days, varying with each transfer. It has never failed to grow progressively and kill when concentrations of at least 5×10^4 cells have been inoculated. The ascitic phase of the tumor does not expand progressively, but appears to reach a maximum relatively early. This phase is followed by rapid infiltration that kills the mouse. The tumor has been passed for more than 32 transfers in the ascitic phase and no change in response to antimetabolites has been observed. Routine transplantation was carried out every 8 to 14 days by intraperitoneal inoculation of 0.1 ml. of whole or diluted ascites.

Mice.—The mice employed in these experiments are derivatives of the C3H/He subline of the C3H strain and include the C3Hf/He, C3H$_e$/De, and C3Hf/Lw sublines (*22*). Subtle differences in behavior of the neoplasm occasionally appear among the different sublines, but the tumor is lethal to all. Neoplasm 70429 does not grow progressively in C3Hf/Bi (Zf) mice, nor in C3Hf/Gs mice, also a derivative of the Bittner C3H subline. The mice were maintained 6 to 8 to a cage and fed Derwood pellets and water *ad libitum*. They were from 2 to 4 months of age and weighed 20 to 26 gm.

Cell counting.—Suitable dilutions (usually 1 to 4×10^{-3} original ascites)

of cells were counted in 4 chambers of the Levy-Hauser counting chamber (a total volume of 0.4 mm.^{3}). These 4 chambers are ruled into 100 squares and, when desired, Poisson distributions of the cells counted can be determined. The cells were withdrawn from the upper-left quadrant of the animal and placed in chilled Locke's solution. They were inoculated within 30 minutes. The dilution technique was performed by serologic methods with serologic pipettes.

Methods of studying cells.—Pathologic material was fixed in Tellyesniczky's fluid and stained with hematoxylin and eosin. While fresh, the ascitic preparations were examined under the phase microscope concurrently with the cell-counting procedure. If the material was contaminated by more than an occasional bacillus or if more than 15 percent of degenerating cells were present, the preparation was eliminated from use. Ascitic smears were stained with Wright's stain.

Preparation and inoculation of azaserine,[4] *DON,*[4] *and other antimetabolites.*—These compounds were routinely prepared in 0.2 *M* phosphate buffer at *p*H 6.0 and were stored in solution when not in use at −20° C. Fresh azaserine was made up every 3 to 5 days and DON every 10 to 14 days. Neither compound was observed to lose its potency, but this could have been obscured by the length of time involved and number of treatments.

All compounds except 6-mercaptopurine were prepared so that a volume of 0.01 ml. was given for each gm. of body weight. These substances, as all other compounds employed, were inoculated subcutaneously in the interscapular region. Azaserine was given 6 times weekly—1 treatment each day. DON was given 5 days each week. Treatment was begun 24 hours following the inoculation of neoplastic cells.

N-Methylformamide was obtained in the liquid state and was diluted and given in daily doses of 100 mg. per kg. 6 times weekly. The first dose each week was 300 mg. per kg. This compound was toxic to the mice; toxicity usually appeared unexpectedly, with the result that groups of mice were found dead. Autopsies revealed hemorrhage in the liver. Amethopterin [5] (methotrexate) was given every 2 days at a dose level of 3 mg. per kg.; 6-mercaptopurine [6] was given daily at dose levels of 75 mg. per kg. 6 times weekly.

Technique of total-cell counts.—The technique for obtaining total-cell counts of the ascitic fluid was accomplished by a washing procedure. The animals were sacrificed by ether anesthesia, and, following this, 6 to 8 ml. of Locke's solution was injected into the peritoneal cavity with a 22-gauge needle. The skin was then removed from the trunk of the animal. The remaining hair was removed by washing under the tap. This produced contractions of the abdominal muscles and general massaging of the peri-

[4] The authors wish to express their appreciation to Dr. Alex Moore and Dr. Harry Crooks of Parke, Davis & Company, Detroit, Mich., for their generous cooperation in supplying azaserine in the L and D forms and also DON.

[5] Amethopterin was kindly supplied by Lederle Laboratories Division of the American Cyanamid Company, Pearl River, N. Y.

[6] 6-Mercaptopurine was kindly supplied by Burroughs-Wellcome & Co. (U. S. A.) Inc., Tuckahoe, N. Y.

toneal fluid. The abdominal region was suspended over a beaker and the abdominal wall incised, allowing the viscera to prolapse into the beaker. The viscera and peritoneum were gently washed by agitation in 3 separate beakers containing 20 to 30 ml. of chilled Locke's solution. The collected washings were stored at 4° C. until counted. Marked clearing was noted in the third washing. Following a dilution procedure, the total number of cells in 0.1 $mm.^3$ were counted. The tumor cells can be differentiated in the wet chamber. Poorly defined cells, such as lymphocytes (small, round cells) and histiocytes were not counted; the latter made up an insignificant portion of the total number of cells. The interpretation of resistance and sensitivity, based on the total number of ascites-tumor cells, is made by: *a*) comparing the response in treated animals to that in controls in any single experiment, and *b*) comparing the effects of a given compound on sensitive and resistant variant populations. As a general rule, the cell populations of controls, if allowed to grow 7 days or longer, reach levels of 100 to 1000 $\times$ 10^6 cells. Inhibited populations that are very sensitive do not exceed 10 $\times$ 10^6 in similar periods. Slight resistance is indicated with populations attaining levels of 11 to 40 $\times$ 10^6. Cell numbers of 50 $\times$ 10^6 and greater are interpreted as indicating definite resistance. The results in tables 9, 10, 11, and 12 reveal clear-cut differences in each case when such comparisons are made.

Results

Mode of Tumor Growth Following Intraperitoneal Inoculation (Gross Description)

The ascitic phase of this tumor is first observed in 7 to 14 days, but occasionally later. During the development of the ascitic phase, cells infiltrate into the retroperitoneal nodes and loose tissues of the mesenteric region. Involvement of the superior mediastinal nodes, presumably by lymphatic spread, also occurs. In the female, infiltration in the form of large, round masses often appears in the ovaries; no similar infiltration occurs in the testes. Occasional nodular infiltration of the spleen develops. Rarely, the tumor will fail to infiltrate the peritoneal structures but will make its appearance in an inguinal lymph node or in the subcutaneous connective tissues. In all cases, dissemination is predominantly through lymphatic channels. Microscopic infiltrations have been seen in the lung and kidney. In a rare instance, microscopic involvement of the liver was seen.

Death from this tumor following intraperitoneal inoculation results from infiltrations into several vital organs. The most usual signs associated with death were, in the following order: massive intraperitoneal tumor, producing intestinal obstruction, inanition, and cachexia; superior mediastinal obstruction, with increasing respiratory and circulatory embarrassment; subcutaneous growth, with massive tumor cachexia and subsequent spread to the regional abdominal lymph nodes.

Study of the survival of animals inoculated with this neoplasm indicates

a fairly wide spread of survival time. Pathologic study reveals the reason for this diversity; death, in terms of survival time, is the result of selective infiltration, in contrast to the generalized infiltration that occurs in other mouse lymphomas. Table 1 shows, in individual experiments, the average survival time in days. Inoculation with 1×10^5 cells or more results in death of 90 percent of the animals by the 45th day, and all are dead by the 77th day. It can be seen from these data that there is considerable variation in the ability of comparable doses of cells to kill the animals.

TABLE 1.—*Survival time of animals bearing neoplasm 70429*

Ascitic transfer generation	Cell dose (10^5 *cells*)	Number of mice*	Age of ascites at time of transfer (*days*)	Average survival (*days*)	Range (*days*)
3	1.7	8	10	35.7	(18–45)
7	0.35	8♂	10	19.3	(15–34)
9	1.05	8	11	35.1	(18–77)
9	0.10	8	11	35.0	(26–53)
12	1.72	8♂	9	29.1	(16–64)
13	2.25	16	8	27.6	(13–61)
15	1.35	16	7	20.9	(12–34)
17	0.43	8♀	8	30.6	(19–37)
17	1.8	23	8	34.9	(14–61)
18	1.76	32♂	8	39.9	(27–60)
19	2.6	12♂	15	31.8	(18–40)
21	0.4	16♀	11	41.7	(26–71)
21	0.95	24	16	31.8	(23–54)
22	1.07	14	18	43.7	(30–48)
21	1.52	6♂	16	38.8	(35–44)
23	0.57	8♂	15	37.5	(27–44)
24	0.4	17	9	26.9	(20–42)
28	0.785	8♂	16	22.8	(15–34)
29	0.9	16	16	20.0	(16–32)
33	0.12	8	11	40.5	(29–60)

*Sexes are mixed except where all males (♂) or all females (♀) are indicated.

In individual experiments where mixed sexes were used, and where comparable groups were available, no striking differences were noted between sexes. The most notable characteristic was the lack of uniformity in terms of survival time from transfer generation to generation.

Effect of Cell Dose on Survival Time

On 7 occasions the tumor was titrated by inoculating doses of cells differing by log units. Lymphocytic neoplasms, when titrated by similar techniques, reveal a linear relationship when the log of the cell dose is plotted against linear survival time. A linear relationship was not found with neoplasm 70429. Table 2 shows the results of these 7 titrations.

Effect of Azaserine on Survival Time

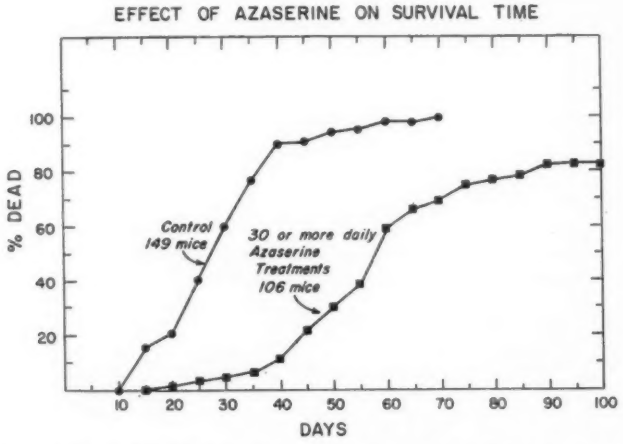
When azaserine was given at dose levels of 2.5 to 5.0 mg. per kg. daily for 6 days each week, the plasma-cell neoplasm 70429 was strikingly inhibited. Inhibition of growth could be maintained for 30 to 40 days

TABLE 2.—*Effect of cell dose on survival time**

Transfer generation	Age of ascitic population‡ (*days*)	Log no. of cells	Average day of death† with following dilutions:					
			10^7	10^6	10^5	10^4	10^3	10^2
3	10	1.7	—	35.7	31.4§	37.7	—	—
9	11	1.05	—	35.1	35.0	33.8	—	—
17	8	4.3	39.3‖	39.3	30.6	35.6	38.6	—
21	16	1.6	26.5	—	24.1¶	—	—	28.6**
22	13	1.52	—	—	38.8	41.1	51	—
30	9	1.5	18.3	—	29.1	—	—	—
33	11	1.27	—	—	40.5	47.0	53.7	—

*There were 2 animals that survived inoculation of neoplastic cells. These were in 2 titration experiments in which 10,500 cells and 1,520 cells, respectively, were inoculated.
†Eight animals in each group. ‡At time of inoculation. §4.5×10^5. ‖2.1×10^7. ¶2×10^3. **8×10^2.

and longer but, in some cases, after the 30- to 40-day period, while treatment with the antimetabolite was continued, palpable tumor or visual ascites developed. The over-all increase in survival time with 30 or more treatments is shown in table 3 and text-figure 1.



TEXT-FIGURE 1.—Effect of 30 or more daily treatments of 5 mg. per kg. of azaserine on survival time of mice bearing the plasma-cell neoplasm 70429 in ascitic form.

It will be noted that 90 percent of the control animals in the 9 experiments were dead by the 40th day, whereas only 11 percent of the azaserine-treated animals were dead from the neoplasm at this time. The steepest portion of the curve is between the 40th and 60th days for the azaserine-treated animals. During this time interval, many animals developed ascites or infiltration of various sites while the course of treatment was continued. Some mice died with advanced tumor during treatment.

It can also be seen that approximately 18 percent of the azaserine-treated mice were alive on the 100th day, and observations were continued on

TABLE 3.—*Effect of long courses of azaserine on survival time of mice bearing neoplasm 70429*

Transfer generation	Cell dose ($\times 10^6$)	No. daily treatments at 5 mg./kg.	Number of controls	Number treated	Average day of death		No. survivors in treated group
					Control*	Treated	
7	0.35	32	8	8	19.3	56.8	0
12	1.72	53	8	8	29.1	71.3	0
13	2.25	30	16	8	27.6	43.7	0
15	1.35	43	16	8	20.9	43.6	2†
17	1.8	30	23	24	34.9	76.1	0‡
18	1.76	59	8	6	43.7	65.5	0
19	2.67	50	12	12	31.8	56.5	0
21	0.95	46	24	13	31.8	97.0	8§
21	0.4	50‖	16	16	41.7	94.0	0¶

*There were no surviving animals among the 131 controls.
†Alive without tumor at 300 days.
‡*See* text-figure 2A.
§*See* text-figure 2C. Eight mice were negative on the 252nd day.
‖2.5 mg./kg.
¶*See* text-figure 2B.

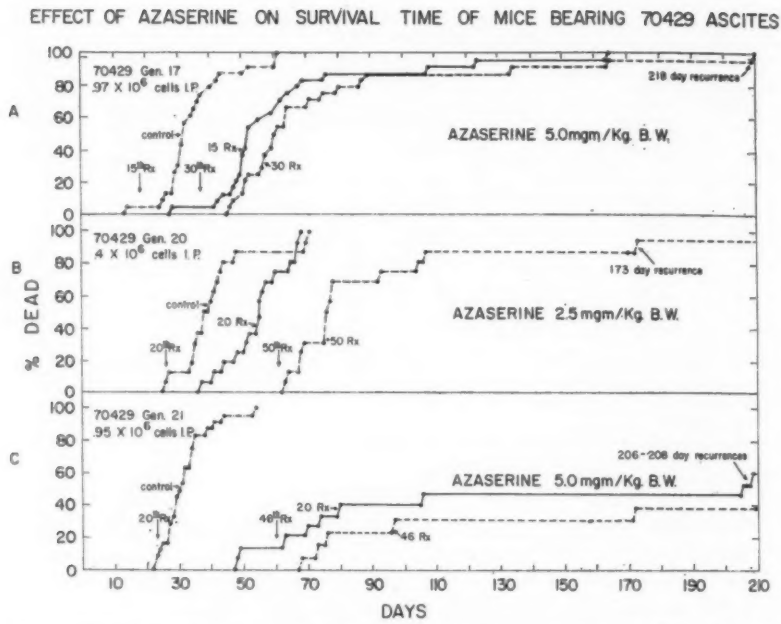
these animals; many developed delayed growths, which were designated "recurrences." In the composite experiments where 15 or more treatments [7] with azaserine were given, there were 173 treated mice and of these 32, or 18.5 percent, lived beyond 100 days. Seventeen of the 32 mice were alive beyond 225 days, but the others died of the neoplasm, as follows: 6 mice died between 100 and 124 days; 1, between 125 and 149 days; 4, between 150 and 174 days; and 4, between 200 and 225 days.

In the first experiment, involving 70 mice (text-fig. 2A), 2 treatment schedules were compared. A short course of 15 daily treatments given 6 times weekly was compared with 30 such treatments. It will be noted that in the treated mice the time to death was retarded and that 6 mice (12.5%) lived beyond 100 days. Five of these mice died of unusual and quite bizarre subcutaneous infiltrations, which in 3 cases were massive subcutaneous infiltrations along the abdominal walls.[8] These unusual subcutaneous infiltrations were typical of the microscopic appearance of neoplasm 70429. In 1 mouse the thickness of the subcutaneous infiltration, which occurred during lactation, was 0.5 to 1 cm. A 218-day "recurrence" showed massive infiltration in the mesenteric node, and there was partial bowel obstruction at the time of autopsy. This subline (70429/218-DR) has been continued as a separate line in transplant.

In another, similar experiment, illustrated in text-figure 2B, where longer courses of azaserine were given, a similar prolongation of life can be seen. In this experiment, the 20th treatment ended on the 26th day and the 50th treatment ended on the 61st day; thus, in each group there were 6 and 11 days without treatment, respectively, before final cessation of treatment.

The late deaths in this group were similar to those previously described,

[7] Text-figure 1 includes only 30 or more azaserine treatments.
[8] One animal was autopsied by Dr. Thelma B. Dunn; all sections were reviewed by her.



TEXT-FIGURE 2.—Survival time of mice bearing plasma-cell neoplasm 70429 in ascites form in 3 individual experiments in which long and short courses of treatment with azaserine were studied. Relationship of appearance of "late recurrences" is indicated. Rx = treatment.

A. Effect of 15 *versus* 30 daily treatments.
B. Effect of 20 *versus* 50 daily treatments.
C. Effect of 20 *versus* 46 daily treatments.

where unusual subcutaneous infiltrations were observed. A 173-day "recurrence" was found with massive infiltration in the cervical lymph nodes. Intraperitoneal transplantation of the neoplastic cells infiltrating the cervical node immediately produced an ascitic tumor, the cells of which were identical morphologically with the stem line of neoplasm 70429. This subline has been designated 70429/173-DR. This recurrent neoplasm was found to be resistant to azaserine; it is discussed in more detail later.

A third experiment in this series, shown in text-figure 2C, reveals similar results. In this case, although the cell dose was in the range of the previous 2 and the survival time of the controls was roughly comparable, inhibition of the tumor with azaserine was particularly striking.

The results of azaserine treatment may then be described, in order of frequency: 1) growth of tumor during treatment, following initial inhibition; 2) tumor growth after cessation of azaserine treatment may occur (*a*) shortly after treatment is stopped, or (*b*) following a prolonged period during which no tumor is detectable; 3) indefinite survival of animals.

In order to demonstrate the specificity of azaserine as a striking inhibitor of neoplastic growth, the D-isomer was employed. This compound

failed to prolong survival of mice in a single experiment. The complete data are presented in table 4.

TABLE 4.—*Comparison of the* D- *and* L-*isomers of azaserine**

	Day of death†								Average
Control	27	29	36	40	40	42	42	44	37. 5
O-Diazoacetyl-D-serine	21	27	32	32	33	33	35	41	31. 7
O-Diazoacetyl-L-serine	45	52	52	54	55	69	82	S‡	58. 4§

*Transfer generation 23 of neoplasm 70429 was used; 0.57×10^6 ascitic cells were used as the inoculum for all groups.

†Each figure represents 1 animal.

‡S = survivor.

§Average of first 7 mice.

Effect of DON on Survival Time

DON also was found to be a powerful growth inhibitor of neoplasm 70429. In one experiment DON was administered at a dose level of 0.375 mg. per kg. daily, 5 times weekly. This dose level was, however, quite toxic and the treatment schedule was interrupted at times to allow recovery. Occasionally, 3- and 4-day intervals passed without treatment with no undue effect. Also, during the initial 2 weeks of treatment with this and lower dose levels of DON, marked weight loss and mild diarrhea occurred, requiring interruption of treatment. Following a rest and resumption of treatment, increased tolerance of these animals to DON was observed. It has been found, however, that the dose level of 0.125 mg. per kg. is very effective in suppressing tumor growth and that prolonged administration at this level is possible.

Table 5 indicates the over-all experience with DON. The prolongation of life was greater with DON than with azaserine, and there were many more survivors.

TABLE 5.—*Effect of DON on survival time of animals bearing plasma-cell neoplasm 70429**

Transfer generation	Cell dose ($\times 10^6$)	No. treatments	Dose (*mg./kg.*)	No. mice		Average day of death		No. treated mice dead	No. survivors	No. days observed
				Control†	Treated	Control	Treated‡			
19	2. 6	34	0. 375	12	9	31. 8	93. 2	4	5	268
22	1. 0	54	. 250	14	8	43. 7	188§	2	6‖	240
22	1. 0	54	. 125	14	7	43. 7	94. 5	4¶	3	240
24	0. 4	15	. 125	18	16	26. 9	75. 5	16	0	212
24	0. 4	20	. 125	18	11	26. 9	81. 8	8	3**	212
24	0. 4	30	. 125	18	9	26. 9	88. 2	8	1	212
28	0. 78	62	. 125	8	8	22. 8	125. 0	7	1	155

*DON given 5 times each week, consecutively.

†There were no survivors in the control group.

‡Average does not include surviving mice.

§Two mice died, on the 139th and 238th days, respectively.

‖One positive animal at 240 days.

¶Two mice, dead at 159 and 196 days, respectively, were not autopsied. These are not included in computing average day of death.

**One mouse dead 212th day with tumor; 1 other has tumor (*see* text-fig. 3).

Text-figure 3 illustrates the comparative action of 3 different dosage schedules. Late recurrences of the tumor in DON-treated mice have been found. Two such lines have been isolated and studied by the technique described for counting the total number of ascitic-tumor cells. These 2 sublines are designated 70429/237-DR and 70429/159-DR.

Study of Resistant Sublines of 70429 (Table 6)

A. Isolation of Resistant and "Recurrent" Sublines

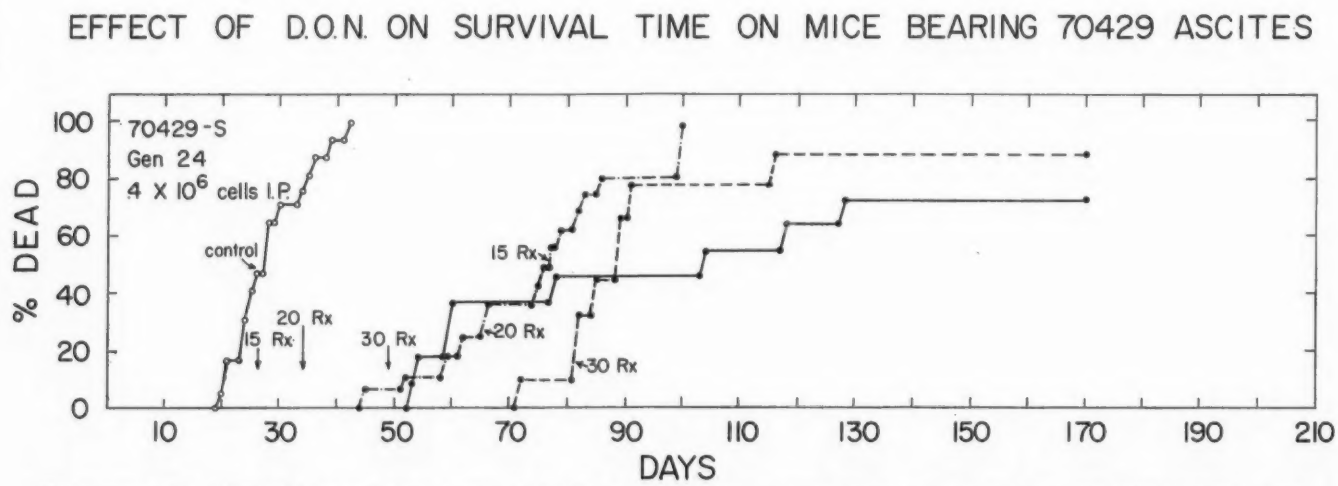
Azaserine-resistant line 1 (70429/AZ-R-1).—This subline was derived during an experiment in which each animal was inoculated with 1.72×10^6 cells of the 12th transfer generation of the ascitic neoplasm. On the 68th day of the experiment, in the group of mice treated with 2.5 mg. per kg. of azaserine 3 of 8 mice were still living, and 1 of these had developed ascites. This ascitic population of cells was removed and tested for sensitivity to azaserine. The results are given in table 7. Additional data, obtained by using the parameter of the total number of neoplastic cells, are confirmatory and are described and discussed later. This tumor was again tested at the 10th and 12th transfer generations after successive serial passage without azaserine, and the neoplasm was found to be fully resistant to azaserine and also cross-resistant to DON. It should be noted from table 7 that the neoplasm 70429/AZ-R-1 was exposed to azaserine for 3 successive transfer generations. The growth characteristics and morphology were the same as in the sensitive stem line.

TABLE 6.—*Guide to the derivation of the various 70429 sublines*

Subline	Transfer generation of sensitive line from which subline was derived	Selecting agent	No. transfer generations exposed to agent*	No. days in mouse before transfer / No. treatments†		
				Gen. 1	Gen. 2	Gen. 3
AZ-R-1	12	Azaserine	3	68/56	15/12	16/13
AZ-R-4	18	Azaserine	1	42/33		
DON-R-1	30	DON	2	51/34	28/19	
NMF-R-1	28	NMF	3	43/36	52/38	22/16
173 DR	20	Azaserine	1	173/50		
218 DR	17	Azaserine	1	218/30		
237 DR	22	DON	1	237/39		
159 DR	28	DON	1	159/59		

*Consecutive generations.

†AZ-R-1 was exposed to azaserine through 3 consecutive generations; in the first, lasting 68 days, 56 daily treatments were given; in the second, lasting 15 days, 12 daily treatments were given, etc.



TEXT-FIGURE 3.—Effect of long and short courses of DON on survival time of mice bearing plasma-cell neoplasm 70429 in ascitic form. Rx = treatment.

Azaserine-resistant line 4 (70429/AZ-R-4).—This subline was derived from a mouse receiving 1.76×10^6 ascites-tumor cells of the 18th ascitic transfer generation. After 33 daily treatments of 5 mg. per kg. of azaserine, this mouse developed a palpable abdominal mass on the 42nd day. The recovered population of cells was used to initiate 70429/AZ-R-4. This line was then passed for only 1 transfer generation in mice receiving azaserine. Tests of the response of this subline to azaserine revealed it to be resistant through the first 5 transfer generations without azaserine, following the 1 exposure to azaserine. When this tumor was tested at transfer generation 29, 10 generations after the first and only exposure to azaserine, it was found to be sensitive to azaserine as determined by the total number of ascites-tumor cells (*see* tables 7 and 12).

DON-resistant (70429/DON-R-1).—This tumor subline was isolated from an animal that had received 15×10^6 ascites-tumor cells of the 30th transfer generation. On the 51st day a mouse was selected from the group that had received 0.125 mg. per kg. daily, 5 times each week. Ascitic cells from the donor mouse were inoculated into mice also receiving DON. During exposure to DON, in the 2nd transfer, there was considerable abdominal swelling at the 28th day, indicating the appearance of a rapidly growing tumor. It was this material that was used to count the total number of ascites-tumor cells (*see* table 10). The DON-resistant line thus was not tested after only a single generation of exposure to DON. Studies on this subline by counts of total ascitic-tumor-cell numbers revealed that it was resistant to azaserine, DON, and NMF.

NMF-resistant (70429/NMF-R-1).—This tumor was derived from a mouse bearing a 28th-generation transfer of the sensitive tumor. This animal had been given 36 daily treatments of *N*-methylformamide, and developed ascites on the 43rd day. This population of cells was then passed for 2 more generations in mice receiving *N*-methylformamide. Mice bearing the second transfer received 38 daily treatments over a 52-day period. The *N*-methylformamide tumor was not tested for resistance after only a single generation of exposure to NMF, but rather after 3 generations of exposure to NMF, using the parameter of total ascitic-tumor-cell count. The results of these studies (*see* table 10) revealed this subline to be resistant to NMF, azaserine, and DON.

The 173-day "recurrence," 70429/173-DR (see *text-fig. 2B*).—The animal bearing this subline was exposed to 50 daily treatments with azaserine. The tumor recurred in the cervical lymph nodes. There was no ascites. At the 173rd day, from the solid tumor, an ascitic population was re-established in the 1st generation by intraperitoneal inoculation of a homogenate of the neoplasm infiltrating the cervical node. This subline of 70429 was subsequently transplanted continuously without exposure to azaserine. When tested by utilizing another parameter of growth, total number of ascites-tumor cells, it was found that this tumor was now resistant to azaserine (*see* table 12).

The 218-day "recurrence," 70429/218-DR (see *text-fig. 2A*).—This subline was isolated from an animal inoculated with 1.9×10^6 ascitic-tumor cells

TABLE 7.—*Response to azaserine of 2 cell lines which developed resistance during treatment with azaserine*

Line	Transplant generation	No. previous transplant generations exposed to azaserine	No. transplant generations passed without azaserine after exposure	No. of mice in each group	Cell dose ($\times 10^6$)	Azaserine dose (*mg./kg.*)	No. azaserine treatments	No. DON treatments	Average day of death		
									Control	Azaserine	DON
70429-S origin	12	0	—	—	1. 72	2. 5	53	—	29. 1	65. 1	—
AZ-R-1*	13	1	0	8	2. 3	2. 5	14	—	25. 6	17. 7	—
	14	2	0	6	0. 65	5. 0	14	—	22. 0	20. 0	—
	18	3	3	8	0. 77	5. 0	17	—	37. 3	24. 5	—
	20	3	5	6	1. 05	5. 0	25	16	41. 0	31. 5	29. 2
				6	0. 0001	5. 0	39	37	51. 8†	47. 6	51. 4
70429-S origin	18	0	—	—	1. 76	5. 0	59	—	43. 7	65. 5	—
AZ-R-4‡	19	1	0	6	Homog.	5. 0	16	—	48. 8	20. 1	—
	20	2	0	6	0. 16	5. 0	18	—	42. 1	22. 8	—
	21	2	1	6	0. 27	5. 0	15	12	37. 8	19. 6	35. 5
	21	1	2	6	0. 03	5. 0	15	—	28. 1	24. 5	—
	24	1	5	6	1. 35	5. 0	27	20	49. 1	36. 0	32. 0

*AZ-R-1 was exposed to azaserine during transplant generations 12, 13, and 14. Thereafter, it was carried without azaserine.

†Average based on 5 of 6 mice; there was 1 survivor.

‡AZ-R-4 was exposed to azaserine at generation 18. In 2 cases, generations 20 and 21, there was a second generation of exposure. These experiments are included to amplify the data. The main tumor line of AZ-R-4 was established from only the single generation of exposure.

of the 17th transfer of the sensitive line. The mouse was given 30 daily treatments of azaserine, ending at the 37th day. The "recurrence" was detected as a mass in the abdominal lymph nodes 181 days after the last treatment. It became an ascitic tumor in 1 transfer generation, and was morphologically identical with the sensitive 70429 stem line. This subline, when tested by the total number of ascites-tumor cells was found to be resistant to azaserine and DON and sensitive to amethopterin (*see* table 12).

The 237-day "recurrence," 70429/237-DR.—This subline was isolated from an animal inoculated with 1.07×10^6 ascitic-tumor cells of the 22nd transfer generation of the sensitive line. The mouse was given 39 daily treatments of DON at 0.125 mg. per kg. each day, ending on the 80th day. The "recurrence" was detected as a huge abdominal mass infiltrating the loose mesenteric and nodal tissues. There was no involvement of the liver, spleen, or kidney. A homogenate of this solid mass gave rise, immediately on transfer, to an ascitic tumor identical with the stem line. When this subline was tested for its response to the several analogues, it was found to be completely sensitive to azaserine, DON, *N*-methylformamide, and amethopterin (*see* table 12).

The 159-day "recurrence," 70429/159-DR.—This subline was isolated from an animal inoculated with 0.78×10^6 ascitic-tumor cells of the 28th transfer generation of the sensitive line. This mouse was given 59 daily treatments of DON at 0.125 mg. per kg. each day, ending on the 88th day. The "recurrence" was detected as a subcutaneous infiltration in the left axillary region and was localized in this region. It was inoculated intraperitoneally as a homogenate and immediately produced an ascitic tumor. It, too, was morphologically identical with the stemline tumor, as determined histologically by Wright-stained smears of the ascitic population. Studies with this neoplasm revealed, by the parameter of total number of ascites-tumor cells, that it was resistant to DON and partially dependent upon azaserine. That is to say, under the conditions of the experiment reported in table 12, animals receiving azaserine contained a greater number of neoplastic plasma cells than the controls. This subline was likewise sensitive to amethopterin.

B. Description of Azaserine, DON, and NMF Resistance

The growth characteristics of neoplasm 70429 make it an unusually good tool for studying the origin of resistant sublines. The reasons for this are the chronicity of tumor growth, the relative lack of toxicity of azaserine and DON at growth-inhibitory levels, and the apparent ease with which resistant sublines can be isolated. The resistant population reveals itself by active growth in the face of continuous treatment with the antimetabolite or by the appearance of active growth long after the treatment has been discontinued, *i.e.*, by a "late recurrence." Two parameters were used to determine whether the tumor was resistant or sensitive. The first parameter was that of survival time (*see* table 7). If animals bearing a given tumor failed to outlive the control animals and developed

tumor ascites or infiltration more rapidly than, or as rapidly as, the control animals, the tumor was considered resistant. The second parameter employed was the use of the total number of ascites-tumor cells. In this case, if the total number of tumor cells gave evidence of great increases, as in the untreated controls, or even exceeded the controls, the subline was considered resistant. The absolute comparison was made by the observed response of the sensitive line to the antimetabolite in question (*see* tables 10 and 12).

In each case where resistance to azaserine or DON was observed by either parameter, cross-resistance to the other compound was also observed (*see* tables 10 and 12).

It is difficult to judge the number of resistant tumors arising during continuous treatment, since not all tumors that grow during azaserine treatment have been isolated and studied. In general, in experiments where there was prolonged administration of azaserine, it was found that the frequency of appearance of presumed resistant populations was apparently quite high. An example of this presumed high incidence is given in 4 selected experiments in table 8. In these, azaserine was administered for as long as 61 to 84 days. It can be seen that there are different patterns of escape from azaserine. For example, in mice bearing transfer generation 19, growth of the tumor appeared rapidly, whereas with mice bearing transfer generation 30A there was a marked delay. In the over-all experiments involving 52 mice, 42 developed detectable tumors during treatment.

It is possible to state that with prolonged azaserine administration (50 or more treatments), approximately 80 percent of the animals will develop palpable tumors. If these tumors were meticulously isolated and tested for resistance they probably would reveal resistance to azaserine and DON, as did 70429 sublines AZ-R-1 and AZ-R-4. This mechanism of resistance is the most common, *i.e.*, growth of the tumor following initial inhibition in the presence of drug. The second mechanism appears to be the appearance of resistance in a "recurrent" tumor, *i.e.*, in one in which growth appears principally as a result of infiltration long after an apparent "metabolically quiescent" period.

To date only 4 "recurrent" tumors have been tested for resistance to azaserine and DON. Of these, 2 were exposed to azaserine and both were found to be resistant. Two were exposed to DON and only 1 was found to be resistant.

To study the stability of a given resistant population it is necessary to remove the tumor from the selecting agent (azaserine, DON, etc.) and then to subject the tumor to a test experiment. The greatest number of transfer generations after exposure to azaserine in these experiments was 12, in the case of 70429/AZ-R-1. After this long period without exposure the tumor was still completely resistant to azaserine and DON (*see* table 10).

Studies of the development of resistance after a single generation of exposure with subsequent removal of the selective pressure, as was observed

TABLE 8.—*Development of palpable tumor during azaserine treatment*

Generation	Cell dose ($\times 10^6$)	Total no. azaserine treatments	Day of last treatment	Average day of death (*controls*)	Range (*days*)	Total no. mice	Number of positive mice* on following days:													
							16–20	21–25	26–30	31–35	36–40	41–45	46–50	51–55	56–60	61–65	66–70	71–75	76–80	81–85
19	2.6	50	66	31.8	18–40	12	0	0	3	5	7	9	11	11	11	11	12	—	—	—
21	0.4	50	61	41.7	26–71	16	0	0	0	1	2	3	4	6	6	9	—	—	—	—
30A	0.15	72	84	29.1	19–43	8	0	0	0	0	0	0	2	5	5	5	5	5	5	8
30B	15.0	72	84	18.3	17–20	8	0	0	0	1	1	1	5	5	5	5	5	5	5	5
29	0.9	72	84	20.2	16–24	8	0	0	0	0	1	5	5	5	7	7	7	7	8	8

*Animals were examined by palpating the abdomen for the presence of a mass.

with the "recurrent" sublines and with AZ-R-4, revealed that AZ-R-4 (azaserine), 173-DR (azaserine), 218-DR (azaserine), and 159-DR (DON) all had developed resistance following exposure to the drug for a single transfer generation. Subsequent studies have not yet been carried out. However, 70429/AZ-R-4 was observed at 1, 5, and 10 generations following exposure and was found to be resistant in the 1st and 5th generations after removal, but it had reverted to sensitivity when tested at the 10th transfer. Reversion had occurred between the 5th and 10th transfer generations. Reversion to sensitivity did not occur with AZ-R-1, where there were 3 generations of exposure to azaserine before removal. An explanation for this reversion to sensitivity may be that sensitive cells were carried along with the resistant tumor and that these sensitive cells, during the period of transfer, in the absence of the selective agent, outgrew the resistant cells. It remains to be seen whether sensitive cells will appear and displace the resistant cells in the "recurrent" tumors upon continued passage without azaserine or DON, as apparently happened with AZ-R-4, or whether the selection has been complete, as demonstrated with AZ-R-1.

The DON (70429/DON-R-1) and *N*-methylformamide (70429/NMF-R-1) resistant sublines were not studied for response to the various antimetabolites after only a single transfer generation and, therefore, it cannot be stated from the data available that resistance occurred in a single transplant generation.

A notable exception to the behavior of the 3 "late recurrences" found resistant to azaserine and DON was 70429/237-DR (DON), which was found to be sensitive to azaserine, DON, and NMF.

In summary, resistance to the compounds studied here can develop by 2 mechanisms. It can develop in the neoplasm that is proliferating rapidly following initial inhibition in the presence of azaserine or DON, or it can develop in a "late recurrence," in which demonstrable growth has been suppressed for more than 100 days. Resistance to azaserine in a single transfer generation occurred in the 70429 sublines AZ-R-4, AZ-R-1, 173-DR, and 218-DR, and resistance to DON occurred in subline 159-DR. This pattern of resistance differs from those previously observed in this laboratory with amethopterin and certain purine antagonists (*see* Discussion).

Total Number of Ascites-Tumor Cells

To establish the observations made with survival-time data, and to extend these with more exacting observations on tumor-cell growth, the action of the various antimetabolites on the total number of ascites-tumor cells was determined. This technique offered, in addition, another advantage in that antimetabolites such as amethopterin, which produced severe toxicity after extended administration, could be studied for their effects during shorter periods of time. The total-cell counts confirmed and extended the observations based on the criterion of survival time. Thus it was also possible, with this parameter, to obtain data regarding

the inhibitory effects of NMF. NMF proved to be an effective inhibitor of neoplasm 70429 and produced a prolongation of life similar to azaserine although, in contrast, no survivors were observed. The results of 2 attempts to establish the effect of NMF with the criterion of survival time revealed, in the first experiment in which 8 mice were used in each group, an average survival time of 22.8 days for the controls and 59.0 days for the treated mice. The treated mice had received 62 daily treatments of NMF. In a second experiment, in which 8 controls and 16 NMF-treated mice were used, the controls died at 19.7 days, the treated group at 60.3 days. There were, however, 8 deaths resulting from drug toxicity. Thus, it can be seen that NMF, too, is a potent inhibitor of neoplasm 70429.

Survival-time data for this compound were often rendered uninterpretable as a result of deaths due to the sudden appearance of hepatic toxicity; thus the data on the number of ascitic-tumor cells, as given later, was very valuable. In numerous experiments with amethopterin and 6-mercaptopurine, survival-time data were unreliable also because of the appearance of toxic phenomena in the treated mice before the death of control animals. Information obtained from total number of ascitic-tumor cells was very valuable in studying the response of this tumor to these antimetabolites. Two striking findings revealed by this technique were the high degree of sensitivity of neoplasm 70429 to amethopterin and its complete natural resistance to 6-mercaptopurine.

Periodic determinations of the total number of ascites-tumor cells revealed rapid, early proliferation of cells, which appeared to attain a maximum number by the 11th day. Thereafter, further striking increases in cell number were not observed. This type of growth response is similar to the findings of Klein and Révész (*23,24*), in which the Ehrlich tumor, as well as several lymphomas, attained a maximum level, after which cell growth seemed to level off.

Many of the present experiments were run on separate days in an attempt to correct for discrepancies created by slowing of growth (table 9). Table 10 summarizes the results obtained with 4 lines of neoplasm 70429. It was found that the sensitive line of neoplasm 70429 was more sensitive to DON than to azaserine. Also, it was sensitive to NMF and amethopterin but resistant to 6-mercaptopurine. Azaserine-resistant line 70429/AZ-R-1, which had been carried for 12 transfer generations without azaserine after resistance to the drug had been observed, remained fully resistant to azaserine and DON, but, strikingly, this tumor was also found to be resistant to NMF. The behavior of the DON-resistant tumor was similar. The azaserine-resistant tumor was similar to the sensitive tumor in its response to amethopterin and 6-mercaptopurine.

An NMF-resistant subline of neoplasm 70429 was developed in the hope that it might shed light on the observed resistance of an azaserine-resistant subline to NMF. This subline (70429/NMF-R-1), as shown in the results, developed only partial resistance to NMF but was strikingly resistant to azaserine. Resistance to DON was also noted. An explana-

TABLE 9.—*Effect of azaserine on the total ascitic-tumor-cell number*

Generation	Cell dose ($\times 10^6$)	Day of cell count	No. treatments	Total number of ascitic-tumor cells (*millions*)									
				Control					Azaserine				
				1	2	3	4	5	1	2	3	4	5
38	0.90	7	5	300	220	210	190	20	3.2	<0.8	<0.8	<0.8	<0.8
32	2.65	8	7	840	680	600	520	—	22.0	6.9	3.8	1.3	—
35	0.64	9	7	720	720	600	500	320	35.0	25.0	16.0	11.0	0.9
37	2.50	11	10	840	620	500	400	—	6.8	1.7	1.7	1.6	1.6
38	0.90	11	8	1060	920	700	580	440	5.2	2.5	2.5	2.5	1.7
32	2.65	14	11	1020	780	440	400	—	43.0	33.0	14.0	9.0	—
38	0.90	15	12	720	620	600	460	400	74.0	48.0	44.0	7.6	0.8

TABLE 10.—*Response of various lines of neoplasm 70429 to certain antimetabolites*

Tumor line	Cell dose ($\times 10^6$)	No. transfer generations since exposure to antimetabolite	Day of cell count	Total number of neoplastic cells in ascitic fluid ($\times 10^6$)															
				Untreated controls					Azaserine (*5 mg./kg.*)						DON (*125 mg./kg.*)				
70429 Sensitive	2. 65	—	9	840	680	600	520		3. 8	1. 3	6. 9	22			4. 4	3. 5	1. 5	1. 0	
	2. 65	—	13	1020	780	440	400		43	33	14	9			6. 8	2. 5	0. 5	0. 3	
	3. 0	—	7	480	380	320	3												
	3. 0	—	9	880	460	420	380												
	0. 7	—	8	580	500	480	320												
	0. 7	—	12	860	800	440	60												
Azaserine-resistant line 1 (AZ-R-1)	2. 05	10	8	780	700	600	500		740	340	320	300			340	320	220	2	
	2. 05	—	12	920	840	840	760		460	380	380	260			360	260	140	—	
	1. 8	12	10	740	560	500	500	380	400	360	300	280	260	160					
DON-resistant (DON-R-1)	0. 8	0	10–11	800	640	600	580	400							500	480	280	260	260
	0. 8	2	10	980	680	680	600	540	780	680	600	500	420		320	300	180	140	120
N-Methylformamide-resistant (NMF-R-1)	0. 9	0	9	88	57	7	—		37	24	2	—			117	54	13		
	—	—	13	340	320	140	—		360	180	120				140	120	11		
	1. 1	1	9	420	360	260	220		160	140	140	80			19	18	17	11	
	—	—	12	440	380	380	240		520	420	380	—			32	—	—	—	

TABLE 4.—Continued

Tumor line	Cell dose ($\times 10^6$)	No. transfer generations since exposure to antimetabolite	Day of cell count	Total number of neoplastic cells in ascitic fluid ($\times 10^6$)		
				N-Methylformamide (*100 mg./kg.*)*	6-Mercaptopurine (*75 mg./kg.*)	Amethopterin (*3 mg./kg.*)
70429 Sensitive	2.65	—	9			
	2.65	—	13			
	3.0	—	7	15 14 6 4		
	3.0	—	9	18 11 10 5		
	0.7	—	8		580 460 400 340	3.8 1.9 0.6 1.2
	0.7	—	12		620 600 400 380	4.1 2.7 2.0 2.0
Azaserine-resistant line 1 (AZ-R-1)	2.05	10	8			
	2.05	—	12			
	1.8	12	10	260 180 80 70 50 40	760 660 640 500 480 260	2.8 2.6 1.6 1.6 1.7
DON-resistant	0.8	0	10–11	230 220 210 120 110	460 420 420 400 29	2.5 2.1 1.6 0.6 0.6
	0.8	2	10	160 160 140 80 40		
N-Methylformamide-resistant (NMF-R-1)	0.9	0	9	11 2 1		4.3 2.6 0.9
	—	—	13	180 160 140		1.2 1.6 —
	1.1	1	9	280 100 80 3		
	—	—	12	80 60 60 3		

*A dose of 300 mg. was given in place of 100 mg. every 7th day.

tion for such cross-resistance is not clear and must await further biochemical data regarding the site of action of NMF.

In an attempt to determine the level of resistance to azaserine, the azaserine-resistant tumor 70429/AZ-R-1 was tested in the 31st transplant generation for its response to daily doses of 5, 10, and 15 mg. per kg. of azaserine. This resistant line was isolated originally from an animal receiving 2.5 mg. per kg. daily. It was transferred twice in the presence of 5.0 mg. per kg. and, prior to this experiment, it had never been exposed to daily dose levels higher than 5 mg. per kg. Table 11 summarizes the results of this experiment. It can be seen that there is equal resistance at all pharmacologic levels of this drug. The development of resistance to azaserine appears to be quite different from that previously reported in lymphocytic neoplasms treated with amethopterin in that resistance to azaserine is acquired in 1 transplant generation. Resistance is complete to very high levels of antimetabolite—in this particular subline to levels as high as 15 mg. per kg. (105 mg./kg., total dose).

TABLE 11.—*Response of 70429/AZ-R-1 (azaserine-resistant) to various dose levels of azaserine**

Daily dose (*mg./kg.*)	Total amount azaserine administered (*mg./kg.*)	No. cells† ($\times 10^6$)				
Control	—	840	760	760	600	360
5	50	420	420	380	340	200
10	100	460	440	280	200	180
15	105‡	420	420	380	300	240

*The initial dose was 1.8×10^6. Transfer 31, *13* generations since exposure to azaserine, was used.
†Each value represents 1 animal.
‡There were only 7 daily treatments at this level due to toxicity.

The ascites-tumor cell-count technique was used to evaluate the response of the various recurrent and resistant sublines described (table 12). It may be noted from tables 10 and 12 that resistance to NMF was never as striking as with azaserine or DON. Yet, it was observed that when there was resistance to NMF, there was marked cross-resistance to azaserine. These findings indicate that whatever selects resistance to NMF also is involved in selecting resistance to azaserine. However, the resistance to NMF is not complete; that is, NMF is still capable of producing some inhibition of the 70429/NMF-R-1 subline. Azaserine resistance in 70429/-NMF-R-1 is quite marked. It is possible that NMF and azaserine are capable of selecting the same type of variant. Also, NMF may be involved in another inhibitory reaction to which resistance has not developed in 70429/NMF-R-1.

Discussion

The findings with neoplasm 70429 may be summarized as follows: This plasma-cell neoplasm, which originated in the inbred C3H strain, follow-

TABLE 12.—*Response of 70429 sublines exposed to azaserine or DON for only 1 transfer generation*

Subline of 70429	Selecting agent	Cell dose ($\times 10^6$)	Transfer generation*	Day of cell count	Total number of ascitic-tumor cells (*millions*)			
					Control	Azaserine (*5 mg./kg.*)	DON (*0.125 mg./kg.*)	*N*-Methyl-formamide
173-DR	Azaserine	1	22 (2)	7	680 550 450 260 —	110 100 90 60		
				12	630 630 580 320 —	400 360 240 220		
		3.8	23 (3)	12	820 660 480 440 —	300 160 120 40		
218-DR†	Azaserine	0.6	20 (3)	10	260 140 120 80	60 40 40 20		
		1.6	21 (4)	12	800 600 560 340 27	330 310 280 240 190	110 108 25 20 17	
159-DR†	DON		30 (1)	12	181 75 74 40 26	220 164 145 80 62	110 76 23 16 1.8	50 48 37 16
237-DR†	DON	1.27	24 (1)	11	490 390 210 200 110	7.0 7.0 4.7 4.0 1.0	6.4 5.5 4.5 1.6 1.5	14 11 11 9.6 2.6
AZ-R-4	Azaserine	2.2	29 (10)	8	400 520 520 40	6.5 4.2 1.8 1.7 1.7 1.8 4.2 6.5‡		

*Number in parentheses indicates number of transfer generations since exposure to azaserine.
†Although not recorded, 218-DR, 159-DR, and 237-DR were sensitive to amethopterin.
‡Ten mg. per kg.

ing 20 subcutaneous passages was converted to a stable ascitic tumor. Cells inoculated intraperitoneally produce a new ascitic population and also infiltrate the mesentery and disseminate through lymphatic channels to regional and distant sites. The end result of this infiltration is death of the host animal. This process of growth may be halted by the action of certain compounds, which have been shown to inhibit purine-ring synthesis. Indeed, the cells that have been introduced and exposed to these compounds may fail to survive and the host animal lives indefinitely. More often, however, growth is smoldering, and the malignant population is contained for extensive periods, only to begin growth again in the presence of the antimetabolites azaserine and DON. If populations are re-established from these recrudescences under the influence of the drugs, growth is no longer inhibited by the very same selecting agents—azaserine and DON. This phenomenon has been called resistance; and our report describes the development of resistance within a single transplant generation. This resistance pattern seems to be similar to the single-step resistance pattern that certain bacteria develop against streptomycin. Sometimes, too, another result occurs with 70429. Growth of the tumor ceases under the influence of the drug, and for a long period—3 months or more—after cessation of drug administration no growth is detectable. Suddenly, rapid growth begins again, this time apparently from a nidus of cells hidden in an abdominal node, such as the cervical nodes, or even at a site distant from the site of inoculation, such as subcutaneous tissues. These new growths are morphologically identical with the original tumor. If they occur in solid tissues, immediately upon peritoneal introduction they become ascitic tumors. Only 4 of these unusual "recurrences" have been studied. Three of these were found to be of the azaserine-DON resistant type, while 1 still responded to the drugs with marked growth inhibition.

The nature of resistance has been described before, with the generalized acute lymphocytic leukemias of the mouse. In this case, resistance occurred after a continued effort to select such a population through many transplant generations. Once the resistant state was established, the new cell types could be removed indefinitely from the selecting agent, and yet their new character was retained. For this reason, it has been considered a genetic event; that is, a new, stable, heritable character has been established in the population. A similar phenomenon has also been observed with the subline AZ-R-1 of neoplasm 70429, where for 12 generations after the last exposure to the drug, the resistant state was preserved within the cells. Though strong resistance was observed after only a single generation of exposure, the cells were transplanted for 2 succeeding generations while administration of the drug was continued. Another line, 70429/AZ-R-4, was exposed for only a single transplant generation and studied for 5 to 10 generations after the single generation of exposure. This subline, though resistant through the first 5 transplant generations, reverted to the sensitive type between the 5th and 10th transplant generations. It is unlikely that physiologic adaptation,

in contrast to genetic adaptation, is the explanation for reversion to sensitivity in this 1 subline. De-adaptation of a population of cells in which adaptive enzyme formation had occurred would most probably be a rapid event (*25*). Reconstitution studies concerned with the role of selection by the drugs employed here on populations of mixed resistant (or dependent) and sensitive cells may provide an explanation of the phenomenon of reversion encountered in 70429/AZ-R-4.

Evidence from the history of 70429 itself may provide a suitable explanation of this phenomenon. During the initial selection of the resistant subline AZ-R-4, it is conceivable that the newly emerged resistant tumor carried with it sensitive cells that, for some reason, were not destroyed by azaserine. This phenomenon is possible, since it has been observed that cells sensitive to azaserine can survive extended treatment schedules (237-DR). By numbers alone, then, the resistant cells dominated the population through the first 5 generations. However, the sensitive cells, which may be postulated to possess a differential growth advantage in the absence of the antimetabolite, subsequently overgrew the resistant forms.

It is impossible at present to define in genetic terms the mechanism concerned with resistance in these populations of cells, since neoplastic cells are somatic cells and, as such, are generally thought not to lend themselves to genetic analysis. However, studies with *Escherichia coli*, strain K-12, have shown that resistance in a single step to high levels of streptomycin results from a single recessive-gene mutation (*26*).

It is to be noted that the level of resistance attained, particularly in subline AZ-R-1, is much higher than that observed during the process of selection for resistance, which is similar to the phenomenon observed in bacteria resistant to streptomycin.

It remains to be demonstrated where the site of action of this heritable character is located. These possibilities must be considered: 1) The resistant cells may (*a*) produce adaptive enzymes, as mentioned above, or (*b*) develop only a hereditary ability to produce these enzymes when the evoking substance is present (azaserine and DON). 2) A genetic character reflected in cell permeability may be involved. 3) The genetic character may be the result of the ability of specific genes to produce new enzymic pathways or a new type of enzyme (*i.e.*, one which does not bind competitively with azaserine and DON). Evidence against the first 2 possibilities is suggested by the facts mentioned above concerning the known method of action of adaptive enzymes and by the observations that *N*-methylformamide, a seemingly unrelated substance, can effectively select for azaserine and DON resistance. Stronger evidence that the site of the genetic change lies within the cells themselves is derived from knowledge of the behavior of azaserine and DON in the pigeon-liver system. In this case, by employing cell-free extracts, DON and azaserine are dynamically capable of competing with glutamine in the conversion of FGAR to FGAM in a very high ratio of competition (*17*). Such observations have been suggested in the work of Greenlees and others with

other mouse tumors (*27*). These problems await the findings of biochemists who investigate resistant and sensitive pairs of these tumors and who will be able to work with cell-free materials.

The synthesis of the purine ring, which has been studied extensively in the pigeon-liver enzyme system has revealed potential sites of action of the powerful metabolic analogues azaserine, DON, and amethopterin. Neoplasm 70429 has been shown to reflect in its growth a marked inhibition by these substances. This tumor, which has both sensitive and resistant sublines, may provide a model system for elucidating these various pathways in neoplastic tissue and for revealing the biochemical pathways of resistance. Such an investigation is currently in progress in this laboratory.

Summary

An ascitic plasma-cell neoplasm (70429), which originated in strain C3H/He mice, has been described. This neoplasm, when inoculated intraperitoneally, attains maximum ascitic growth about the 11th day; thereafter, and concurrently with this phase of growth, there is progressive infiltration into the loose mesenteric tissues and deep abdominal nodes, which ultimately kills the mouse.

The growth of neoplasm 70429, as demonstrated by the accumulation of ascitic-tumor cells in the peritoneal cavity and by the length of life of animals bearing this neoplasm, was markedly inhibited by the glutamine antagonists azaserine (O-diazoacetyl-L-serine) and DON (6-diazo-5-oxo-L-norleucine). A striking increase in survival time (double or triple that observed in control mice) has been observed following prolonged courses of these 2 antimetabolites. Some mice have survived for extensive periods free of the disease. Increases of ascitic-tumor cells of neoplasm 70429 were shown to be markedly inhibited by azaserine, DON, amethopterin, and *N*-methylformamide but not by 6-mercaptopurine.

When mice bearing neoplasm 70429 were exposed to long courses of azaserine (30 to 84 daily treatments) there was an initial period of prolonged inhibition of tumor growth. Following this inhibition approximately 80 percent of the mice developed progressive ascitic and infiltrative growths of the neoplasm.

Resistance to azaserine and DON, which occurred within a single transfer generation, was studied in detail in 5 different sublines. Resistance developed to high levels of azaserine, although the initial selecting dose was considerably lower. The azaserine-resistant state has been maintained in 1 case in the absence of the selecting drug for at least 12 transfer generations, showing that this character is stable and heritable. In another case the azaserine-resistant subline reverted to sensitivity. An explanation of this phenomenon is discussed. For purposes of comparison, and for the study of cross-resistance, sublines resistant to DON and *N*-methylformamide were developed through several transfer generations. Cross-resistance of azaserine-resistant sublines to DON and *N*-methylformamide, of the DON-resistant subline to azaserine and *N*-methylformamide, and

of the *N*-methylformamide-resistant subline to azaserine and DON has been observed without evidence of change in sensitivity to amethopterin and 6-mercaptopurine.

There were 2 growth patterns for emerging resistant populations. The first and more common followed prolonged inhibition while the selecting agent was still being administered. The second followed prolonged inhibition throughout the course of treatment and continued for 2 to 6 months after cessation of the drug. This second type was described as the "recurrence." The late "recurrences" often developed in unusual sites, such as cervical lymph nodes or distant subcutaneous tissues.

The characteristics of the resistant sublines and the possible mechanisms involved are discussed.

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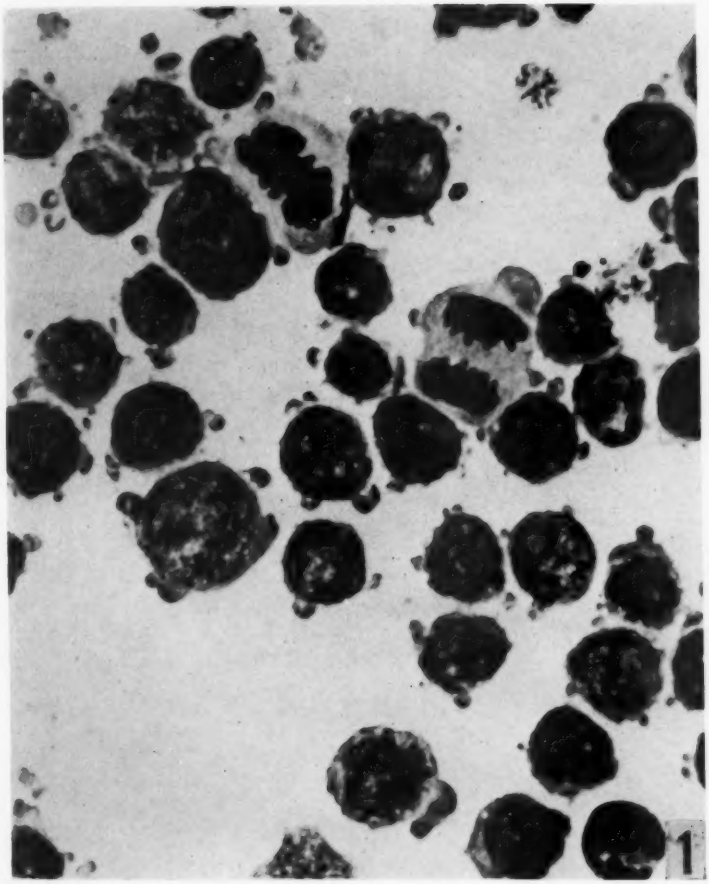
PLATE 30

FIGURE 1.—Smear of fully developed 70429 ascites, stemline tumor. Leishman. × 570



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The Histogenesis of Virus-Induced Chicken Leukemia [1,2]

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Leukemia is regarded as a "systemic" neoplastic disease, involving the hematopoietic system *in toto*. Its pathogenesis has been studied little, however, and the question of a focal origin as opposed to a generalized primary reaction is still unsolved. This is due mainly to difficulties in obtaining adequate material for investigation. Even the Japanese investigations, in which samples of blood and bone marrow were taken at frequent intervals after the atomic-bomb explosions, revealed very little concerning the morphogenesis of leukemia.

Virus-induced leukemia in the fowl offers an opportunity to study in some detail the morphologic events in this disease during its earliest developmental stages. Although some investigations have been made in the past, the data are incomplete and contradictory in several respects. The liver and spleen are probably involved later than the bone marrow (*1*). The delay might depend on an extramedullary colonization of leukemic bone-marrow cells, which does not, however, prove a focal origin. To investigate the onset of leukemia, it is necessary to study the changes in the organ primarily affected—the bone marrow.

Two main questions will be considered: 1) Is leukemic induction a focal reaction of a few cells or a generalized onset of neoplastic growth in the whole hematopoietic system? 2) Are the extravascular granulopoietic cells and the intrasinusoidal erythropoietic tissue affected differently by the leukemia virus?

In studying these questions, a comparison will be made with the regenerative bone-marrow reaction after a single phenylhydrazine injection producing hemolysis. Also, the nonspecific changes caused by the injection of the virus-containing preparation will be checked.

Materials and Methods

White Leghorn chicks between 3 and 5 days of age were used. Leukemia was induced in a group of 35 animals by single intravenous injections into each animal of an equal amount of a cell-free cytoplasmic homogenate prepared from pooled leukemic spleens as described in reference (*2*). The

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[2] This work was supported by the Lotten Bohman Research Fund, by the Swedish Research Group on Bovine Leukosis, and by the Lady Tata Memorial Trust.

virus used was a so-called erythroblastotic strain, characterized by production of a uniform and very acute leukemia with a survival time of about a week after the injection of massive doses (*2,3*). On each successive day following inoculation, blood smears stained with May-Grünwald-Giemsa were made. Starting on the second day, 2 animals were sacrificed daily until the 10th day after inoculation, when the experiment was terminated. The bone marrow in the femur and tibia of both sides was removed as complete and intact as possible, fixed in formol-sublimate-acetic acid, embedded in paraffin, and serially sectioned. Sections from the liver and spleen were also prepared and stained with hematoxylin and eosin. Spontaneous deaths from leukemia occurred during the final 4 days of the experiment. The microscopic findings in these cases were considered together with the findings in the 2 animals sacrificed daily during those final 4 days.

The nonspecific changes accompanying intravenous administration of the homogenate prepared as described above were investigated in a separate experiment. A group of 15 animals was injected with an aliquot of the cell-free homogenate that had been inactivated at 56° C. for 30 minutes (*4*). Another group of 10 animals served as untreated controls. Starting on the first day after inoculation, 2 animals from each group were sacrificed daily and bone-marrow samples prepared as previously described.

The regenerative erythroid reaction was effected by intramuscular injection of the hemolyzing agent phenylhydrazine hydrochloride (50 mg./kg. body weight). These animals were killed 24, 48, and 72 hours after injection. The bone marrow was prepared as described above.

The results were recorded in the following manner: *Bone marrow.*—The morphologic picture after the injection of active virus was evaluated with the semiquantitative criteria in table 1. *Liver.*—To quantitate the results differential counts were made. This was possible because practically no extramedullary hematopoiesis occurred in the chicken liver at the time of hatching. Two different kinds of cells were distinguished—nonleukemic cells (mainly liver-cord cells) and leukemic cells. A total of 300 cells was counted in each liver. The error involved in the counting procedure was established by counting 10×300 cells in the liver from a leukemic chick killed on the 8th day after inoculation. The mean percentage of leukemic cells in this chick was 27.4 percent. The maximum error in a single count of 300 cells was calculated to be 13.3 percent. The percentage of tumor cells for each day was computed as the mean of the figures for the 2 sacrificed animals on a given day plus the figures obtained from the animals dying spontaneously of leukemia on that same day. *Spleen.*—In this organ only unequivocal leukemic infiltration was recorded.

Results

Leukemic Changes

Bone marrow.—Figure 1 illustrates the appearance of a sinusoid from an untreated, normal 5-day-old chicken. After injection of leukemia

virus, the earliest definite changes detected morphologically occurred on the 3rd day. They consisted of small, localized, budlike proliferations of the cells lining the sinusoids (fig. 2). In some areas, the process had extended to the whole circumference of a sinusoid (fig. 3). Such a sinusoid consisted of a thick peripheral layer of leukemic cells. In the center was a core of mature erythrocytes without any transitional forms of the leukemic type of cell. Later this core disappeared and, on the 4th day, several sinusoids were completely filled with tumor cells. In these early stages the greater part of the bone marrow still had a quite normal appearance. Figure 4 shows the relationship between the leukemic portions and the surrounding normal bone marrow. Even in the leukemic portions the extravascular granulopoietic centers were morphologically normal. During the following days (5th–7th day after inoculation) the leukemic process was rapidly extended. The previously formed leukemic areas enlarged until all normal marrow was replaced by leukemic tissue. This replacement was complete on the 8th day after inoculation. In this final stage the trabeculae were compressed and granulocytopoiesis was impaired. No indication of the original localized nature of the leukemic proliferation was found and the disease in this stage involved all parts of the bone marrow (fig. 5 and table 1).

TABLE 1.—*Bone-marrow picture 2 to 10 days after inoculation of leukemia virus*

	No. animals on following day of inoculation:								
	2	3	4	5	6	7	8	9	10
No microscopic changes	2	1	1						
Occasional scattered leukemic foci as shown in figs. 2 and 3		1	1		2				
Approximately 25% of bone-marrow specimens exhibiting leukemic changes				2		1			
Occasional small areas of normal bone marrow surrounded by leukemic marrow						1		1	
Completely leukemic marrow with loss of all normal structure						1	5	4	4

Liver.—The changes in this organ started considerably later than in the bone marrow. First, on the 7th day after inoculation leukemic cells (27%) were found (fig. 6 and table 2). This finding occurred simultaneously with the appearance of unquestionable leukemic cells in the peripheral blood. The first spontaneous deaths also occurred on the 7th day. After this day, the percentage of tumor cells in the liver rose rather slowly, reaching a maximal value of about 50 percent.

TABLE 2.—*Leukemic-cell infiltration in liver after inoculation of leukemia virus*

Day after inoculation	2	3	4	5	6	7	8	9	10
Percentage of leukemic cells	0	0	0	0	0	27	35	45	54

The leukemic cells were located in the liver capillaries (fig. 6). Signs of pressure atrophy of the liver-cell cords could be seen.

Spleen.—Due to the complicated and nonuniform structure of the spleen, leukemic-cell infiltration could not be interpreted quantitatively with ease. The changes seemed, however, to parallel those in the liver, *i.e.*, the first leukemic cells were encountered on the 6th or 7th day after inoculation.

Nonspecific Changes

Such changes were generally absent in the bone marrow, liver, and spleen, as indicated by a comparison between the untreated controls and the animals injected with heat-inactivated homogenate. Only in 1 animal were signs of exaggerated granulocytopoiesis seen. These changes were wholly confined to the trabeculae. The erythropoietic sinusoids were unaffected in all cases.

Regenerative Changes

In sharp contrast with the early leukemic changes was the essentially diffuse involvement of all bone-marrow sinusoids during regeneration after the hemolysis induced by phenylhydrazine. After 12 hours, definite signs of stimulated erythropoiesis appeared. They consisted of an increased number of immature erythroblasts gradually developing into more mature forms (fig. 7). At 24 hours (fig. 8) a great number of erythroblasts filled every sinusoid and immature erythrocytes circulated in the peripheral blood. The diffuse character of this regenerative response in the bone marrow is illustrated in figure 9.

Discussion

Because of the definite spatial separation of erythropoiesis and granulocytopoiesis in chicken bone marrow, the uniform response toward virus-induced leukemia with the strain used (*2*), and the absence of nonspecific reactions the development of leukemic changes could be followed in some detail.

The scheme apparently followed during these changes was characterized initially by focal leukemic changes in the bone marrow that were confined to some of the sinusoids. These foci later extended and finally involved the whole bone marrow. At this late stage, leukemic blood changes began to occur simultaneously with the appearance of leukemic cells in the capillaries of such extramedullary tissues as the spleen and liver. In the latter organ, where the admixture of leukemic cells could be followed quantitatively, infiltration was found to occur rapidly; on the 6th

day no leukemic cells could be detected, whereas 27 percent were found on the 7th day.

The term "erythroblastic" leukemia was used originally to describe the progressive anemia exhibited in many cases and the concomitant appearance of typical erythroblasts in the peripheral blood (*3*). The present results give a more clear-cut basis for this term, since the cells primarily affected were shown to be the erythropoietic stem cells that line the sinusoids.

In general, leukemia has been interpreted as either a neoplastic growth or a systemic, potentially reversible, reactive change of the hematopoietic organs. The sequence of events in the virus-induced leukemia described above conforms with the concept of a malignant neoplasm starting in localized areas of the bone marrow, with no initial blood changes or extramedullary manifestations of the disease.

The etiologic factors of "spontaneous" leukemia in man and other mammals are for the most part still obscure. Some findings, however, indicate a viral factor as the leukemia-inducing agent in mice, in which leukemia was formerly regarded as spontaneous (*5*). Thus, before more experimental data have been collected, the "virus" etiology cannot be excluded, and the pathogenetic events described above may also be valid for some types of leukemia in mammals, including man.

Summary

The histogenesis of a virus-induced chicken leukemia was investigated. The histology of the bone marrow, liver, and spleen showed that the leukemic process started as foci in some of the bone-marrow sinsusoids on the 3rd day after viral inoculation. On the 7th day the bone marrow was diffusely involved and leukemic infiltration of the liver and the spleen had started. Nonspecific cytologic changes were inconspicuous, as shown by the injection in a control group of a heat-inactivated virus preparation. Regenerating bone marrow, in contrast with leukemia, at all stages exhibited a diffuse "systemic" response.

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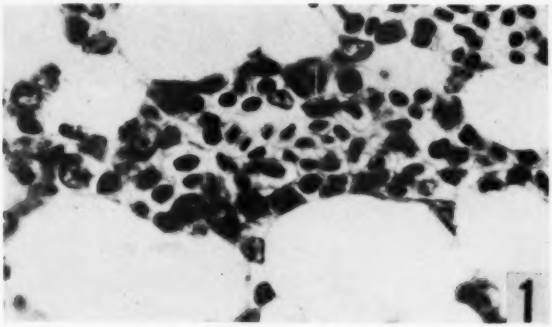
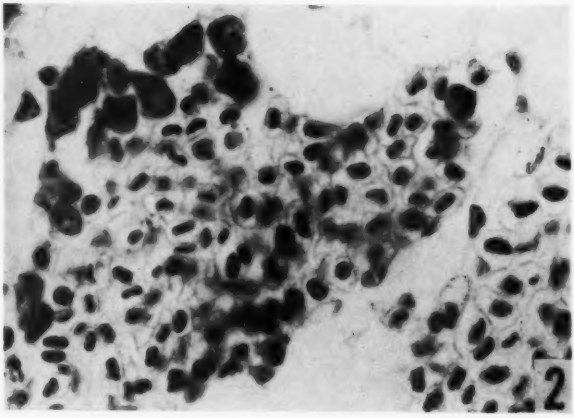
PLATE 31

FIGURE 1.—Normal sinusoid from chicken bone marrow. Hematoxylin and eosin. × 500

FIGURE 2.—Leukemic sinusoid 3rd day after the virus injection. Proliferating leukemia cells in *upper left*. Hematoxylin and eosin. × 500

FIGURE 3.—Leukemic sinusoid 3rd day after virus injection. Most of the sinusoid is composed of leukemia cells. Hematoxylin and eosin. × 500





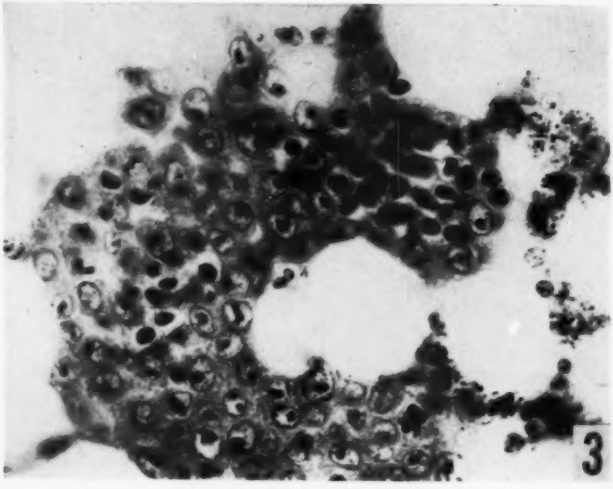


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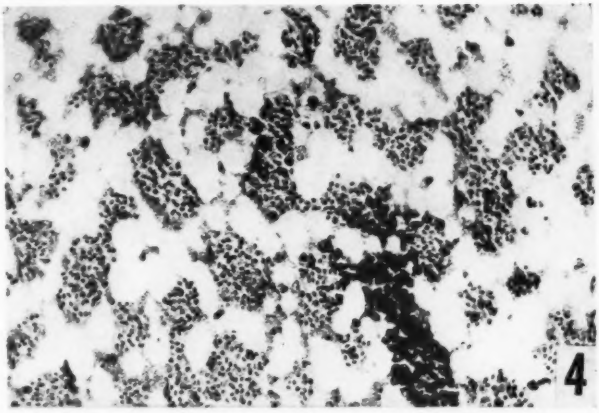
PLATE 32

FIGURE 4.—Leukemic bone marrow 5th day after virus injection, showing the proliferating cells. Giemsa. × 100

FIGURE 5.—Leukemic bone marrow 8th day after virus injection. Very few normal bone-marrow cells. Hematoxylin and eosin. × 500

FIGURE 6.—Leukemic liver 8th day after virus injection. Some capillaries are filled with leukemia cells. Hematoxylin and eosin. × 700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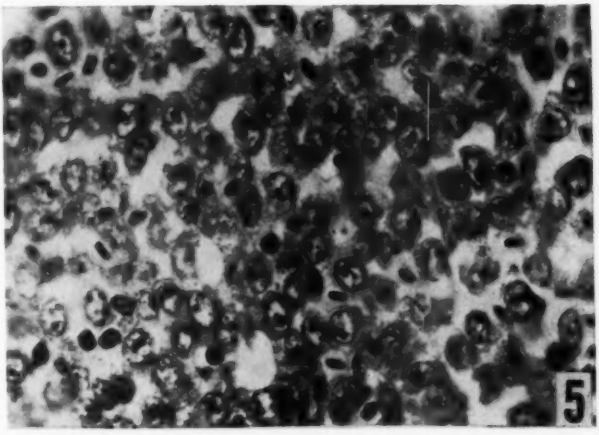
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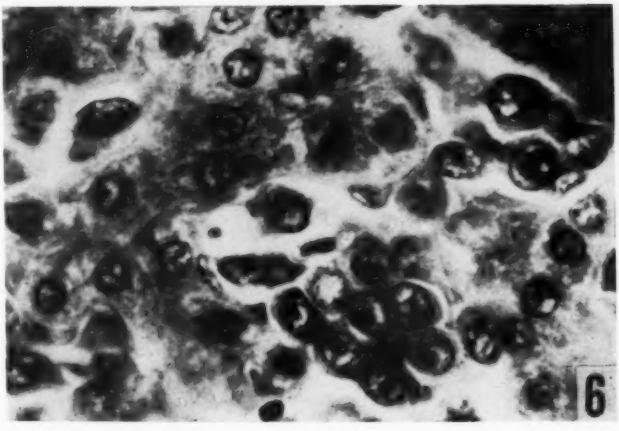
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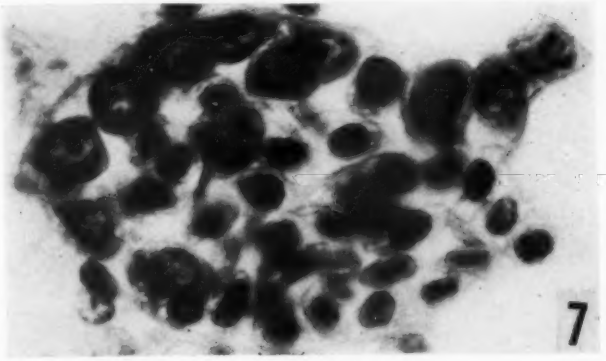
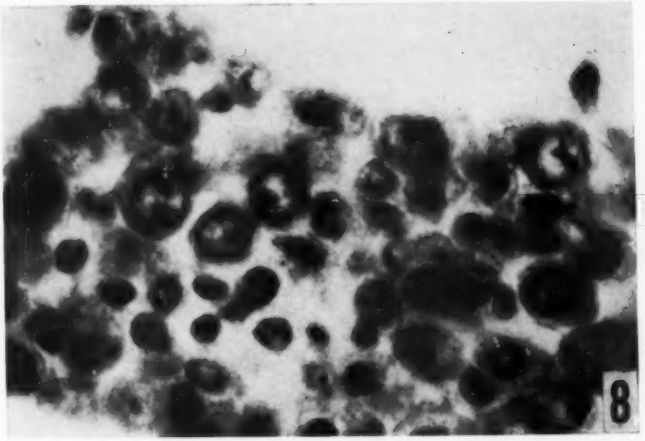
PLATE 33

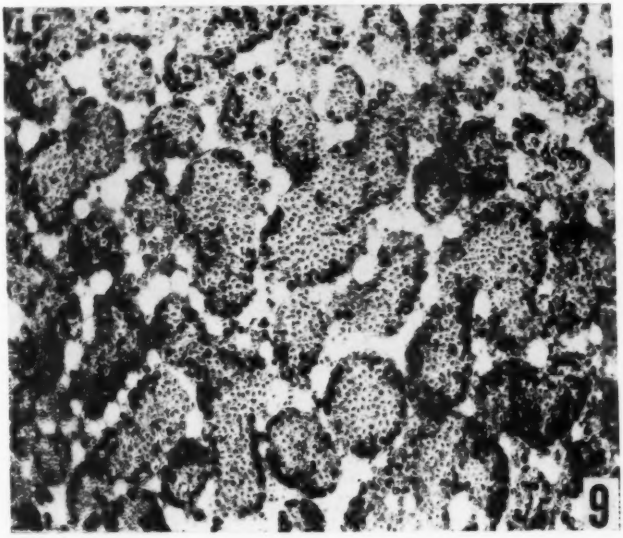
FIGURE 7.—Regenerating sinusoid 12 hours after phenylhydrazine injection. A general reaction of the stem cells at the periphery of the sinusoid. × 900

FIGURE 8.—Regenerating sinusoid 24 hours after phenylhydrazine injection. All stages of erythroblast maturation. × 900

FIGURE 9.—Regenerating bone marrow 12 hours after phenylhydrazine injection, showing proliferating cells. Giemsa. × 100







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Interrelations in Respiratory, Phosphorylative, and Mitotic Activity of Ehrlich Ascites-Tumor Cells: Influence of Sulfhydryl Reagents [1]

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Interrelationships between the respiratory, phosphorylative, and mitotic activity of Ehrlich ascites-tumor cells have been studied *in vitro* by exposing the cells to graded concentrations of agents affecting these activities and comparing the relative changes produced. Previous studies determining the influences of dinitrophenol and stilbestrol indicated that the rate of cell division, as measured by uptake of radioactive phosphorus into deoxyribonucleic acid (DNA), may be independently related to both the respiratory rate and the availability of high-energy phosphate (*1,2*). The studies were extended in the present investigation with determination of the effects of several sulfhydryl reagents.

The importance of sulfhydryl-containing compounds in numerous biological processes, including cell respiration and division, has been demonstrated repeatedly (*3,4*).[4] Much of this information has been gained through detection of changes in the metabolic and functional activities of cells effected by reagents reacting readily with and destroying the sulfhydryl moiety of these compounds. Three such sulfhydryl reagents were employed in the present experiments: *p*-chloromercuribenzoic acid, a mercaptide-forming agent, *o*-iodosobenzoic acid, an oxidizing agent, and nitrogen mustard (HN_2),[5] an alkylating agent.

Materials and Methods

The conditions and procedures used for growth and processing of tumor cells, for measurement of respiration, and for separation and assay of phosphate fractions were similar to those described in preceding reports (*1,2*). Ehrlich ascites-tumor cells, grown in C3H mice about 3 to 4 months old, were harvested after 7 days. Pooled cells from 2 to 5 mice were washed 4 times in Krebs-Ringer-phosphate solution and were uniformly suspended in the same medium to a concentration of 15 to 20

[1] Received for publication November 26, 1957.
[2] *Present address*: National Cancer Institute, Bethesda 14, Md.
[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.
[4] A recent symposium was completely devoted to 1 such compound, glutathione (*5*).
[5] Methyl-bis(β-chloroethyl)amine.

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percent. The packed-cell volume was determined by centrifugation of an aliquot in a Wintrobe hematocrit tube.

Amounts of suspension containing 0.2 ml. packed cells were added to the main chambers of Warburg vessels and were diluted to 2.1 ml. with Krebs-Ringer-phosphate. The suspension was buffered with 0.4 ml. of 0.15 *M* tris to establish *p*H 7.4 at 37° C. When included, sulfhydryl reagents [6] and P^{32} (approximately 25 μC in neutral isotonic solution), in a total volume of 0.5 ml., were tipped in from side arms at zero time, following a 10-minute equilibration period. Central wells contained alkali and fluted filter papers. Temperature was maintained at 37° with air as the gas phase.

Immediately following the respiratory period of 1 hour, the contents of duplicate vessels were combined and centrifuged. The supernatant "medium" was decanted, and both cells and medium were subjected to phosphate fractionation (*1*). Inorganic-phosphorus (IP) and labile-phosphorus (LP) fractions were separated from extracts prepared with cold trichloroacetic acid. Non-nucleotide organic phosphorus, the remainder of phosphorus in the extracts, was characterized as ester phosphorus (EP) and was calculated as the difference between total acid-soluble phosphorus and the sum of IP + LP. Deoxyribonucleic acid phosphorus (DNAP) was separated from the acid-insoluble residue of the cells by a modified Schmidt-Thannhauser partition. All phosphorus contents are shown as μg. per ml. cells, or fluid associated with 1 ml. of cells.

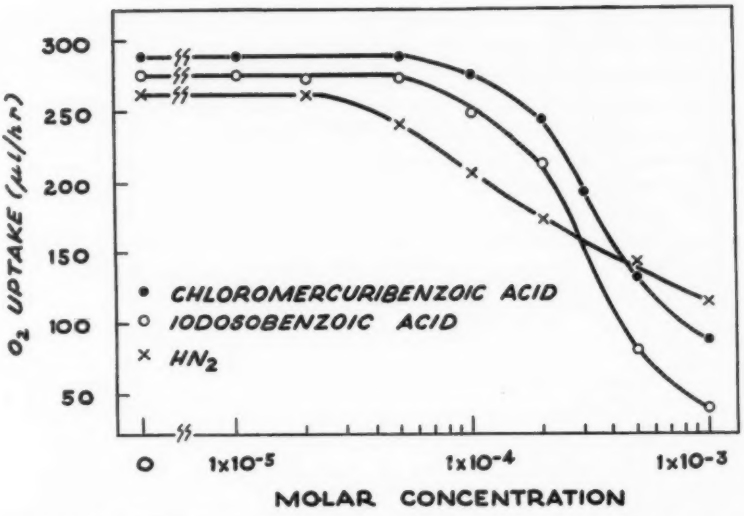
Radioactivity of IP fractions was calculated in terms of specific activity (S.A.), defined as the ratio of percentage counts added to mg. phosphorus per ml. Activity of other fractions is expressed as relative specific activity (R.S.A.), the percentage ratio S.A. of the fraction to that of the corresponding IP fraction.

Results

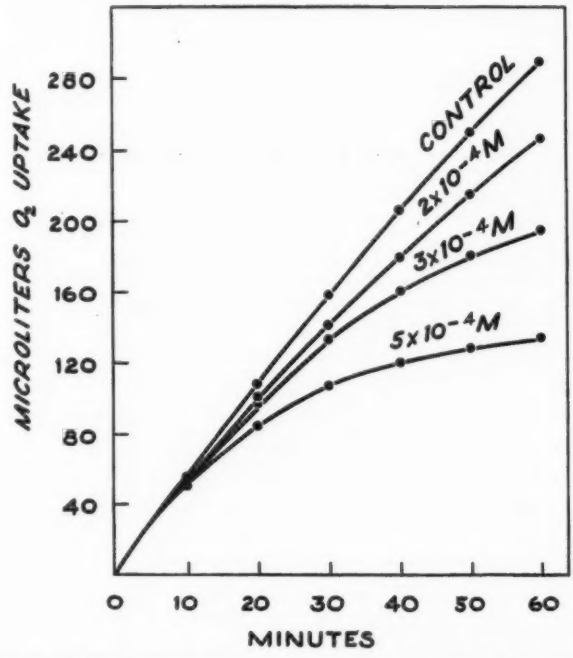
The influence of graded concentrations of the 3 reagents on endogenous-oxygen utilization of the ascites-tumor cells is shown in text-figure 1. The benzoates showed no effect on respiration until a concentration of 1×10^{-4} *M* was reached. With further increase in concentration, respiration fell off sharply. The shapes of the curves resembled those for stilbestrol and quinone, although the inhibitory action of stilbestrol became apparent at a lower concentration (*2*). HN_2 was less effective than the benzoates in inhibiting O_2 uptake, respiration declining gradually with increased reagent concentration. A limited effect of HN_2 on cellular respiration, at least during the first hour of exposure, has been observed with several mammalian tissues (*6*), as well as with unicellular organisms (*7,8*).

As shown in text-figure 2 with chloromercuribenzoate, all sulfhydryl reagents exhibited a delayed action in inhibiting tumor-cell respiration. No inhibitory influence was apparent during the first 10 minutes of

[6] *p*-Chloromercuribenzoic acid and *o*-iodosobenzoic acid were dissolved in minimal amounts of alkali. HN_2 (Mechlorethamine hydrochloride, Merck) solutions were prepared and added immediately prior to equilibration.



TEXT-FIGURE 1.—Effect of graded concentrations of sulfhydryl reagents on O_2 uptake of Ehrlich ascites-tumor cells. Each flask contained 0.2 ml. of packed cells. Reagent concentrations are final levels after tipping in of sidearm contents.



TEXT-FIGURE 2.—Time-rate curves of ascites-tumor-cell respiration as influenced by *p*-chloromercuribenzoic acid.

exposure. This contrasts with the immediate effect on respiration produced by dinitrophenol and stilbestrol.

A difference between the benzoates and HN_2 on metabolism of the ascites-tumor cells was clearly demonstrated in their effects on cellular-phosphorus relationships. Results with chloromercuribenzoate are presented in table 1; iodosobenzoate values were entirely comparable. Nitrogen mustard data are shown in table 2.

TABLE 1.—*Influence of* p-*chloromercuribenzoic acid on phosphorus relationships of ascites-tumor cells*

Mercuribenzoate concentration	IP		LP		EP		DNAP	
M	μg./ml.	S.A.*	μg./ml.	R.S.A.†	μg./ml.	R.S.A.†	μg./ml.	R.S.A.†
Cells								
0	297	76. 2	130	97. 6	515	27. 9	534	0. 172
5×10^{-5}	285	76. 1	122	97. 7	540	21. 9	524	0. 153
1×10^{-4}	266	75. 1	116	95. 3	510	29. 7	529	0. 148
2×10^{-4}	199	75. 0	91	60. 7	595	15. 1	517	0. 075
3×10^{-4}	166	89. 3	35	33. 6	301	15. 5	535	0. 020
5×10^{-4}	117	113. 8	20	10. 0	88	28. 4	531	0. 005
Medium								
0	363	124			74			
5×10^{-5}	370	121			80			
1×10^{-4}	380	123			127			
2×10^{-4}	521	111			154			
3×10^{-4}	683	94			283			
5×10^{-4}	840	85			461			

*Specific activity = percent counts added per mg. P per ml.

†Relative specific activity $= \frac{\text{S. A. fraction}}{\text{S. A. IP}} \times 100.$

TABLE 2.—*Influence of nitrogen mustard on phosphorus relationships of ascites-tumor cells*

HN_2 concentration	IP		LP		EP		DNAP	
M	μg./ml.	S.A.	μg./ml.	R.S.A.	μg./ml.	R.S.A.	μg./ml.	R.S.A.
Cells								
0	326	71. 4	172	88. 4	567	22. 3	529	0. 072
5×10^{-5}	318	77. 1	183	86. 2	541	27. 5	513	0. 062
1×10^{-4}	376	66. 8	146	88. 9	541	37. 1	545	0. 044
2×10^{-4}	420	71. 6	79	79. 7	493	11. 8	496	0. 032
5×10^{-4}	458	64. 7	34	59. 8	508	25. 0	521	0. 027
Medium								
0	321	139			51			
5×10^{-5}	324	131			54			
1×10^{-4}	367	120			44			
2×10^{-4}	355	139			67			
5×10^{-4}	395	133			62			

With increasing concentrations of mercuribenzoate there was little change in either amount or S.A. of cellular IP until levels of reagent inhibitory to respiration were reached. The cellular IP content then began to fall, while the S.A. increased.[7] An opposite change occurred in medium IP; concentrations of reagent inhibitory to respiration increased medium IP levels, while the S.A. fell. Changes in EP concentration of cells and medium were similar to those in IP, higher reagent levels producing decreases in cellular EP and increases in medium EP. Both the concentration and activity of cellular LP diminished with an increase in benzoate concentration, paralleling the fall in respiration. DNAP concentration, as had been noted with other agents, remained unaffected by mercuribenzoate; uptake of P^{32} however, fell markedly with a rise in reagent levels.

Nitrogen mustard, in contrast to the benzoates, produced a rise in cellular IP with increasing concentration; S.A. remained relatively unchanged. Medium IP levels also increased slightly, with the S.A. maintaining constancy. Although the cellular content of EP decreased slightly at the higher reagent levels, medium EP concentration remained stable. Both the concentration and the activity of cellular LP decreased with increasing levels of HN_2, a change which invariably accompanied onset of respiratory inhibition with all reagents studied.

Discussion

Although chloromercuribenzoate and iodosobenzoate act similarly to stilbestrol (*2*) and higher concentrations of dinitrophenol (*1*) in inhibiting respiration of Ehrlich ascites-tumor cells, it is obvious from phosphate exchange that different modes of action are involved. The rise in medium IP which accompanied increase in dinitrophenol concentration always lagged behind and seemed to result from the elevation in cellular IP. There was no overt evidence of alteration in membrane permeability. Similarly with stilbestrol, the cellular content of IP rose with increasing reagent concentration until the very highest level was reached. The subsequent fall in cellular IP occurred simultaneously with manifestations of damage to the cell membrane. The phosphate exchanges which attended increase in benzoate concentrations, on the other hand, were all indicative of impairment in cellular permeability. Indeed, a change in the physical characteristics of the cells exposed to the agents was manifest. It appears likely, therefore, that a major action of the benzoates on Ehrlich ascites-tumor cells may be directed toward the cell membrane.

[7] Mention should be made of a change in physical characteristics of the cells after exposure to mercuribenzoate. Following the respiratory period and centrifugation of suspensions to separate cells from media, typically constant volumes of solidly packed cells were obtained from control preparations or cells exposed to noninhibitory levels of benzoate. With an increase in reagent sufficient to bring on respiratory inhibition, however, the volume of the cellular residue began to increase. Moreover, instead of remaining solidly packed, the cellular centrifugate became viscid and tended to run out of the tube on inversion. At the same time, the concentration of protein in the medium began to increase, as shown by quantitative biuret determination and by an increase in the volume of trichloroacetic acid-insoluble material (unpublished data). Similar effects, of even greater magnitude, were observed after exposure of cells to inhibitory levels of iodosobenzoate. These phenomena, probably denoting damage to the cell membrane and altered cellular permeability, very likely have a bearing on the phosphate changes observed.

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The importance of sulfhydryl groups in maintaining the integrity and permeability of cellular membranes has been demonstrated previously. Barron (*3*) found the oxidation of added substrates by *Mycobacterium creatinovorans* to be inhibited indiscriminately by sulfhydryl reagents and suggested the presence of sulfhydryl proteins responsible for permeability at the cell surface. Shacter (*7*) noted agglutination and partial lysis of yeast cells exposed to chloromercuribenzoate associated with impairment in glucose oxidation. Benesch and Benesch (*9*) observed that sheep erythrocytes were hemolyzed by organic mercurials, probably through reaction with cellular sulfhydryl groups essential for maintenance of the intact cell structure. Sheets *et al.* (*10*), in demonstrating the hemolysis of human erythrocytes by chloromercuribenzoate, called attention to the delayed reaction between the reagent and cellular sulfhydryl groups. Hemolytic action could be reversed by washing the cells as long as 30 minutes after contact with the agent. In the present experiments a delayed action of the agent on respiratory inhibition of Ehrlich ascites-tumor cells was noted.

Nitrogen mustard, a compound readily alkylating sulfhydryl groups, had an action on the ascites cells more closely akin to that of dinitrophenol and stilbestrol than to its sister sulfhydryl reagents. The agent, however, is known to react with many cellular components (*11*). It would appear that under the present experimental conditions HN_2 affected cellular loci other than membrane sulfhydryl groups (*cf. 8,11*).

In spite of the diverse mechanisms apparently involved in the actions of agents on respiration and phosphorus relationships of Ehrlich ascites-tumor cells, in all cases inhibition of respiration was associated with a decrease in concentration and R.S.A. of labile phosphorus, as well as with lowered uptake of P^{32} into DNAP. The relationship of cell division to respiratory rate and availability of high-energy phosphate previously suggested therefore still pertains.

Of the 3 agents used in the present experiments, only HN_2 has been employed extensively *in vivo*. The compound is effective in restricting ascites formation and in increasing survival time of mice bearing the Ehrlich carcinoma (*12,13*). Its action *in vivo* thus seems to relate to the *in vitro* action of HN_2 on Ehrlich ascites cells in inhibiting both uptake of P^{32} into DNAP, observed in the present experiments, and incorporation of glycine-2-C^{14} into DNA adenine, demonstrated by Le Page and Greenlees (*14*). On the other hand, Davidson and Freeman (*15*), studying the influence of prior administration of HN_2 on P^{32} uptake *in vivo*, found that the agent had no effect on P^{32} incorporation into tumor DNA of mice bearing adenocarcinoma 755, although growth of the neoplasm is inhibited slightly. Moreover, Heidelberger and Keller (*16*) noted only slight inhibition of P^{32} uptake into tumor DNA *in vitro* after administration of HN_2 to rats bearing Flexner-Jobling carcinoma, a neoplasm markedly affected by the drug. Therefore, no distinct relationship is apparent at present between the biochemical actions of HN_2, either *in vitro* or *in vivo*, and the inhibitory action of the drug on tumor growth.

Summary

The influence of graded concentrations of sulfhydryl reagents on interrelations between respiration and phosphorylations of Ehrlich ascites-tumor cells was studied *in vitro*. Associated with inhibition of cellular respiration by *p*-chloromercuribenzoic acid and *o*-iodosobenzoic acid, there was a change in the physical characteristics of the cells and marked outpouring of phosphorus, suggesting damage to the cellular membrane. Nitrogen mustard had a lesser effect in inhibiting respiration and did not appear to affect cellular permeability. Inhibition of respiration was in all cases accompanied by decrease in concentration and specific activity of labile phosphorus and in lowered uptake of radioactive phosphorus into deoxyribonucleic acid.

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Mammalian Chromosomes *in Vitro*. VII. Heteroploidy in Human Cell Strains [1,2]

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It has long been a recognized fact that polyploidy and heteroploidy frequently occur in neoplasms, both *in situ* and *in vitro*. Available data indicate that this conclusion may also be applied to human material, although it has been studied less extensively than that of the mouse and the rat. Koller (*1*) reported a hypodiploid rectal carcinoma, Hsu (*2*) presented a case of a subtetraploid sarcoma, and Hansen-Melander *et al.* (*3*) observed a subtriploid ovarian cystocarcinoma. Recently, Koller (*4*) and Levan (*5*) have contributed more examples of chromosome anomalies in human cancers. In a case of melanoma in which the sample consisted of cells suspended in ascitic fluid (*2*), the chromosome number fell within the diploid range after the cells had grown for a few days *in vitro*. This observation may be questioned because there were numerous nonmalignant elements present in the original fluid that might have contaminated the chromosome counts.

During recent years a number of cell strains have been established from both malignant and nonmalignant human tissues. Hsu (*6*) studied the chromosomes of strain HeLa and found that it was a hypotetraploid. Recently, Moore *et al.* (*7*) and Levan (*5*) mentioned that the numbers most frequently encountered for Chang's liver and conjunctiva strains were in the 70s. Since both strains are the progeny of normal tissues, the phenomenon of heteroploidy in these lines poses an important question: Are most or all of the established strains heteroploid, regardless of their origin? If so, what are the implications? The present article reports data regarding chromosome numbers of 8 human cell strains.

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Material and Methods

The following strains were employed in this study:

Neoplastic tissues

1) S-3—This is a clonal strain derived by Puck and Marcus (*8*) from Gey's strain HeLa, carcinoma of the cervix.
2) KB—An epidermoid carcinoma of the floor of the mouth, established by Eagle (*9*) in December, 1954.
3) Maben—Adenocarcinoma of the lung, established by Frische *et al.* (*10*) in June, 1953, from a pleural effusion.
4) Oregon J-96—Derived by Osgood and Brooke (*11*) in April, 1954, from the circulating blood of a patient with monocytic leukemia.

Nonmalignant tissues

5) Ch-L—Chang's liver strain (*12*) established in 1954. For convenience, we suggest the symbol Ch-L for this strain to distinguish it from any future strain of similar origin.
6) Ch-C—Chang's conjunctiva strain (*12*), similarly designated by us to avoid confusion. The tissue was removed from a child in February, 1954. It was considered nonmalignant (*13*).
7) McCoy—This strain was derived by Pomerat (unpublished) from cells of the synovial fluid in the knee joint of a patient suffering from degenerative arthritis in October, 1955. Details of the strain characteristics will be described by Dr. Pomerat elsewhere.
8) FF—Also an undescribed strain established by Pomerat and his associates from the sole of the foot of a fetus in November, 1956.

All the strains were maintained in stock bottles with no plasma clot. The fluid medium consisted of 45 percent human ascitic fluid, 50 percent Gey's balanced salt solution, and 5 percent chick embryonic extract. Preparations for chromosome studies were set up in roller tubes with regular plasma-clot and fluid medium. After 6 to 8 days of incubation at 37° C., the cultures were treated with a hypotonic solution, fixed in Helly-Zenker's or in methanol, and stained with May-Grünwald-Giemsa.

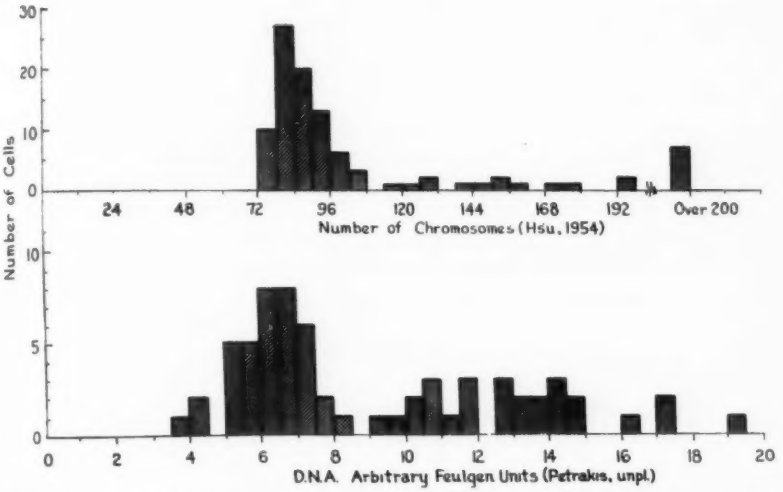
In most cases the errors in counting did not exceed 2, except for strain J-96 where the error might be slightly larger. At least 50 cells were counted for each strain to obtain a sample indicating the distribution of chromosome numbers. However, only 20 cells were available for strain J-96.

It should be added that fragmentation of the chromosomes in the cells studied was practically nil, for we had paid attention to the presence of centromeres. Structural changes of chromosomes, however, such as extremely long chromosomes suggestive of translocation, have occasionally been found.

Results

Before presenting the data from the present study it would be of some value to cite the chromosome counts of strain HeLa made by one of

us (*6*) for comparison, since strain S-3 is a clonal derivative of the original HeLa. According to Hsu, HeLa was hypotetraploid, with the modal chromosome number in the 80s. Distribution of deoxyribonucleic acid (DNA) values, measured from individual nuclei in HeLa made by Dr. Nicholas L. Petrakis (personal communication to TCH), showed remarkable agreement with the actual counts. The 2 sets of data are graphically reproduced in text-figure 1. Minor discrepancies between the 2 sets of data did occur, and they have been discussed in detail (*14*).



TEXT-FIGURE 1.—Comparison of chromosome counts and DNA measurements on individual nuclei of the HeLa strain.

Distributions of chromosome-number classes of the various strains are presented in table 1. Since counting errors were not entirely avoidable, grouping 5 chromosomes in each class would probably represent more closely the nature of each cell line.

It is a striking fact that all 8 strains examined in this study were heteroploid. The modal chromosome numbers in some strains were in the 80s, and in others, in the 70s or 60s. The diploid number, whether it is 48, conventionally accepted for man for many years, or more likely 46, recently claimed by Tjio and Levan (*15*) and by Ford and Hamerton (*16*), was either completely absent or represented by a negligible number of cells—not more than a few percent of the total count. Similarly, there was a negligible number of tetraploid cells. Even in the younger strains, such as McCoy and FF, which were not more than 6 months old when the preparations were made and were supposedly of normal origin, drastic changes of chromosome constitution seemed to have taken place already. Observations on Henle's intestine strain 407 and leukemia strain Oregon J-111 (Osgood) also revealed their heteroploid nature, although no exact counts have been made.

TABLE 1.—*Frequency distribution of chromosome numbers in 8 human cell strains*

Strain	36–40	41–45	46–50	51–55	56–60	61–65	66–70	71–75	76–80	81–85	86–90	91–95	96–100	101–105	106–110	111–115	116–120	121–125
Neoplasms																		
S-3						1		1	16	20	10	1						
Maben					1		1	4	11	20	5	3	2					
KB						1	1	4	6	17	14	5						
J-96		1		1	2	2	6	3	1	1	1		1					
Normal																		
Ch-L	1		1		2	4	12	28	20	15	5	1	5	1	1			
Ch-C			1		2	4		4	10	12	2	2	3	2	2		1	
McCoy	2				7	24	38	21	4						1		1	
FF					1	6	29	18	1						1			

TABLE 1.—Continued

Strain	126–130	131–135	136–140	141–145	146–150	150	Average *s*	Average *s* in relation to the chromosome number of man*		Total cells counted
Neoplasms										
S-3						1	82	Hypotetraploid	4 × −10	50
Maben						3	82	Hypotetraploid	4 × −10	50
KB				1		1	83	Hypotetraploid	4 × −9	50
J-96						1	69	Triploid	3 ×	20
Normal										
Ch-L				2		2	77	Hypertriploid	3 × +8	100
Ch-C	1	1				2	82	Hypotetraploid	4 × −10	50
McCoy						2	67	Hypotriploid	3 × −2	100
FF	1	1					69	Triploid	3 ×	58

*Diploid human chromosome number considered as 2*n* = 46.

Comparing strain S-3 with its mother line, HeLa, it is rather obvious that the 2 strains possessed similar stemline elements. However, S-3 showed a much narrower spectrum than HeLa. In S-3 most of the cells fell in the 80s, whereas in HeLa practically all the classes had some representatives. According to Levan (*5*), both Ch-L and Ch-C showed a high frequency of chromosome numbers in the 70s. Our results on strain Ch-L agreed with his observation. Strain Ch-C seemed to contain more cells in the 80s, but samples in both Levan's paper and in the present study are too small to warrant any definite conclusion.

Little attention was paid to the chromosome morphology in the present investigation. A minor difference seemed to exist between the strains of malignant origin and those of nonmalignant origin; namely, the chromosomes of the latter group were more easily spread by the hypotonic solution than those of the former. This was partly due to extensive stickiness of the chromosomes in the cancer lines. However, the distinction was not clear-cut, for many cells in the strains of nonmalignant origin also showed stickiness. In contrast, we have noticed that very few cells in primary cultures of any nonmalignant tissues showed such a phenomenon. Cells showing C-mitotic appearance were common in both types of strains. Figures 1 to 4 depict a few selected cells used in this report.

Discussion

It has been the experience of most tissue-culture workers who have attempted to establish cell strains that the original cellular growth from *in situ* tissues is generally good. As time goes on, after several generations of subculturing or if the cultures are left in the original containers after many changes of the medium, the culture may go into a stage of sluggish growth. At this time mitotic activity is at a very low rate, and many cells die. It appears that the "strain" is not capable of survival and the worker usually becomes discouraged. Whether one nurses such cultures assiduously or permits an atmosphere of negligence, suddenly one may find that the cells have regrown in one or more of the containers. After this stage, if proper care is made in subculturing, the strain is easily established. It is also of interest to note that in some cases cells of connective tissues, originally spindle-shaped, with loose, tongue-like cell alignment, may become somewhat flattened and lose their typical cellular arrangement.

When the chromosomes are examined after the strains have been established, as reported in this paper, the cells exhibit extensive heteroploidy, with very few or no euploids. Even in more recently established cell lines, such as McCoy and FF, whose chromosomes were examined approximately half a year after the primary cultures had been made, nearly all the cells showed heteroploid constitution. This would not be surprising for strains of malignant origin since their primary cultures may already contain a high proportion of cells with abnormal chromosomal numbers. In "normal" strains, however, the finding of heteroploidy would imply profound changes during the early period of cultivation,

because primary cultures of normal tissues always show a very high percentage of diploid elements (*15,17,18*).

Tissue-culture media and conditions must be regarded as abnormal in a strict sense. Even though they can support migration and mitosis of cells, *in vitro* conditions fail in certain ways to duplicate the *in vivo* environment. Cellular relationships, for instance, obviously deviate from normal. Many attempts to establish cell strains have failed, even with embryonic material. Probably the normal cells (presumably diploid) cannot survive and proliferate for long periods of time *in vitro*, and the cell population gradually loses its reproductive ability, whereas heteroploids, with their special combinations of chromosomes and therefore special genotypes, may adapt to the new environment. It is of interest to cite the work on rats by Makino and Tanaka (*19*), which showed that regenerating cells *in situ* contained a relatively high frequency of heteroploids after partial extirpation of liver. When tissues are set up in culture vessels, trauma is unavoidable. This may stimulate some production of abnormal mitoses and consequently abnormal chromosome numbers. During the early periods of cultivation, abnormal mitoses may repeatedly take place, thus increasing the frequency of heteroploids. As the vigor of the diploids finally subsides, the stage of "sluggish growth" is reached. This period is probably very important in the history of a strain, for it may represent the stage of transformation. Heteroploids, which are fewer in number, now begin to dominate the population and replace the diploids. Vigorous growth is apparent after this transition era due to the tremendous rate of mitosis in the new type of cells. In other words, the phenomenon of strain establishment is likely to be a result of selection within a cell population. Cultures failing to develop into cell strains may contain no heteroploids or may contain heteroploids with a genotype that does not possess an adaptational advantage over the diploids. It is possible also that the culture media used might be one of the factors influencing the spectrum of heteroploidy.

The foregoing discussion is not a mere speculation. Evidence of such transformation has been obtained from 1 strain, originally from a nonmalignant tissue. It was followed from the second subculture to the eighth subculture, at which stage the strain became totally heteroploid (unpublished data). However, many more cases of such generation-by-generation analysis are needed to fortify the hypothesis of selection.

Moore *et al.* (*7*) have demonstrated that Chang's liver and conjunctiva strains (Ch-L and Ch-C in our terminology) produced tumors when injected into animals or patients. This means that the strains have become malignant during the 2 years in the *in vitro* environment. It seems that a change to malignancy is associated with the changes in chromosome numbers, or *vice versa*. It would be of great interest to conduct experiments to demonstrate that the transition period from diploid to heteroploid precisely coincides with the change of tumor-producing ability.

It must be borne in mind that the data and discussion presented in this

article are restricted to these fast-growing strains only. Gey *et al.* (*20*) stated: ". . . of special interest here is the behavior of some of the normal cell strains, some of which have remained stable for periods up to almost 10 years; while others have changed greatly over a short interval during the prolonged cultivation." A number of the unchanged strains are slow growers that are maintained by meticulous attention in contrast to the ease with which faster lines may be carried. Efforts to detect whether the slow strains retain their diploid constitution will indeed be valuable, for such information would be of cardinal importance to the hypothesis of adaptability of heteroploids and possibly malignant conversion *in vitro*.[5]

One conclusion can be safely drawn from the study of chromosome patterns of the cell strains, *i.e.*, none of the so-called "normal" strains is truly normal, at least as far as chromosomal constitution is concerned. Any *in vitro* experiment requiring normal cells should employ primary cultures and refrain from using such cell strains.

Summary

Eight cell strains, 4 of neoplastic and 4 of nonmalignant origin, were studied with respect to their chromosome numbers. All the strains proved to be heteroploids with very few diploids and tetraploids, or none at all.

It is postulated that heteroploids are more able to adapt to the *in vitro* condition than the diploids and tetraploids. Perhaps after a number of generations of subculturing, the heteroploids, originally in the minority, supplant other cell types and become the new stem line.

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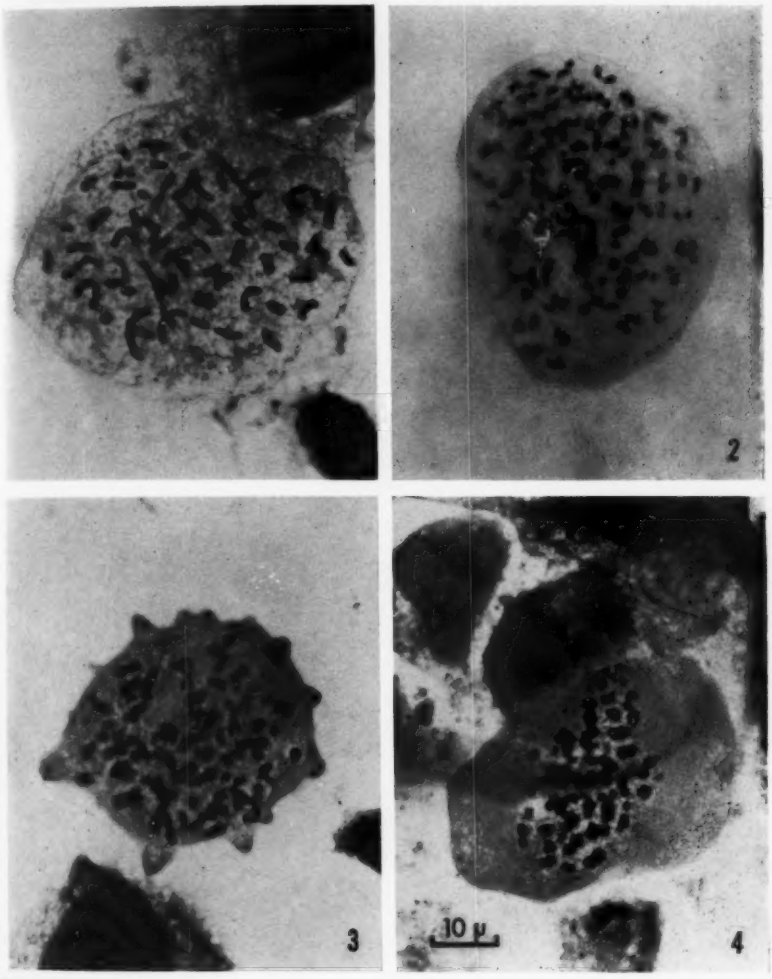
PLATE 34

Chromosomes of cells from 3 strains, all stained with May-Grünwald-Giemsa. For magnification see scale on figure 4.

FIGURE 1.—A cell from strain McCoy; 76 chromosomes. Methanol fixation.

FIGURE 2.—A cell from strain KB, showing "C-mitotic" appearance. Methanol fixation.

FIGURES 3 AND 4.—Two cells from strain Ch-C. Helly-Zenker's fixation. Figure 3, a cell with 83 chromosomes. Figure 4, overcondensed chromosomes.



Hsu and Moorhead

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Induction of Mammary Tumors by Injection of *N*-2-Fluorenylacetamide into Artificial Cecal Pouches in Rats[1,2]

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The experiment to be described was designed for the purpose of producing polyps and carcinomas of the large bowel. Tumors of the large bowel of the rat are rare. Polypoid lesions have been produced by injection of motor lubricating oil or by various diets (*1*). In the observers' opinion, however, these lesions were inflammatory rather than neoplastic. Insertion of methylcholanthrene pellets into the blind ends of colostomy preparations and injection of methylcholanthrene into the wall of the colon were unsuccessful (*2*). In a group of 385 rats, 7 instances of adenocarcinoma of the rectum were induced in males by Spitz and her associates (*3*) after weekly subcutaneous injections of benzidine. The results of Walpole *et al.* with 3,2′-dimethyl-4-aminodiphenyl were more successful (*4*).[4] Of 23 rats of both sexes, 21 developed multiple tumors of the small and large intestines following weekly subcutaneous injections. A few large-bowel lesions have been encountered following the use of *N*-2-fluorenylacetamide (*5–8*). In the experiment reported here, repeated injections of *N*-2-fluorenylacetamide were made into cecal pouches in an attempt to achieve high concentrations of the carcinogen in the large bowel and to minimize the formation of hepatomas and other remote visceral tumors. We failed to induce bowel lesions, but breast tumors developed in many animals.

Materials and Methods

Eighty-two female Sprague-Dawley rats were used. The animals were fed a commercial laboratory chow supplemented once a week by canned horse meat and brewer's yeast. The animals were caged in pairs. The cages had perforated floors, which prevented additional contact with any carcinogen contained in the feces. Water was given *ad libitum*. The cecal pouches were prepared by splitting the lower abdominal musculature

[1] Received for publication November 14, 1956.

[2] Work done while in the Department of Pathology, College of Medicine, University of Utah, Salt Lake City, Utah.

[3] The authors wish to express their appreciation to Madeleine Spjut for her technical assistance.

[4] 3,2′-Dimethyl-4-aminodiphenyl was not used as the carcinogen in our experiment because the experiment was already planned and under way at the time the report of Walpole *et al.* was published.

at the mid-line through a skin incision. A portion of the cecum was sutured to the abdominal muscle and fascia. Four or 5 interrupted black silk sutures were enough to fix the cecum in place. The skin was then closed over the herniated cecum (fig. 1). The pouches measured between 3 and 4 cm. in diameter.

N-2-Fluorenylacetamide (manufactured by Eastman Kodak Co.) was dissolved in 50 mg. per ml. of polyethylene glycol 300. The rats were divided into 3 age groups: 42 rats received weekly intracecal injections of 1 ml. of *N*-2-fluorenylacetamide beginning at the age of 3 months, a second group of 13 rats was started at the age of 4 months, and a third group of 15 at the age of 5 to 6 months. Ten control animals received 1 ml. of the vehicle. Two animals, without treatment or operation, were kept as weight controls. Three animals, 2 from group I and 1 from group II, were discarded when they died of cecal cutaneous fistulae after receiving less than 8 injections. Sacrifices, in groups of 4, were made at 3-month intervals. Two control animals were sacrificed at the same time as 4 experimental animals. At monthly intervals, the animals selected for sacrifice were put to death after the last dose of carcinogen so that any delayed effect of carcinogen could be studied. Autopsies included gross inspection of all viscera, neck organs, tumors, and the cecal pouch. Histologic sections were made of each of these unless cannibalization or severe autolysis interfered.

Results

The first breast tumors appeared after 15 weeks of injections (table 1). Most often the tumors enlarged progressively to form ulcerating masses measuring up to 6 cm. in diameter. Occasional tumors regressed and reappeared at a later time; a few remained stationary until death. Multiple tumors were common (table 2). The tumors early became fixed to the skin and ulcerated; none were fixed to the thorax. A few invaded skeletal muscle. The breast tumors were usually well-differentiated adenocarcinomas with extensive necrosis. Less well-differentiated areas were common. Small peripheral lobules of non-neoplastic breast tissue, frequently hyperplastic, could be seen in many instances. The tumors were histologically similar, regardless of the time of appearance, number of

TABLE 1.—*Time of appearance of tumors*

Week	No. of rats with tumor	Week	No. of rats with tumor	Week	No. of rats with tumor	Week	No. of rats with tumor
15	2	24	5	33	1	42	2
16	1	25	2	34	3	43	0
17	5	26	1	35	0	44	0
18	2	27	0	36	2	45	0
19	3	28	8	37	0	46	1
20	3	29	1	38	1	47	0
21	2	30	1	39	0	48	0
22	2	31	3	40	0	49	1
23	0	32	1	41	0		

TABLE 2.—*Multiple tumors*

No. of tumors....	1	2	3	4	5	6
No. of rats.....	20	15	8	7	2	1

injections, or age of the animals. One animal had pulmonary metastases. This animal had received a total of 34 injections; the first breast tumor appeared after 17 weeks.

Very few tumors of other tissues were found. There were 2 hepatomas, 1 epidermoid carcinoma of the external ear, and 6 salivary-gland tumors. The carcinoma of the external ear was locally invasive. One histologically benign, hyperkeratotic skin lesion occurred in the milk line; no tumors of the large bowel, cecum, or the skin over the cecal pouch were found. No tumors or other atypical epithelial changes were found in other portions of the gastrointestinal tract.

The livers showed frequent foci of inflammation in the lobules and periportal areas and occasional focal necrosis. One liver displayed extensive periportal fibrosis and focal necrosis of the parenchyma. This animal had received 38 injections of the carcinogenic agent; 2 breast tumors were present.

No breast tumors were found in the first group of 4 rats when injections were discontinued after 14 weeks and the animals were sacrificed at monthly intervals thereafter. The 4 rats of the next group that received 26 injections developed breast tumors. Additional breast tumors appeared in 3 of the rats after cessation of the injections. The only single tumor was first noted 2 months after the last injection. In the later groups the animals died with large ulcerating tumors before the course of injections was completed; the animals that did not develop tumors died of pneumonia.

The age at the onset of injections appeared to be an important factor. Of the 40 rats 3 months old when the injections began, 35 (86%) developed breast tumors; 25 had multiple tumors. In the 4-month group, 10 (80%) of the rats had tumors, 5 single and 5 multiple; 8 (53%) of the rats in the 5-to-6-month group developed tumors, 5 single and 3 multiple. The rats that were free of tumors received the carcinogen until death. This time range was from 24 to 45 weeks. One control animal developed a breast tumor, histologically a fibroadenoma.

Comment

Our goal of producing epithelial lesions of the large bowel was unsuccessful. Instead, 49 of 67 rats (73%) that received weekly intracecal injections of *N*-2-fluorenylacetamide developed mammary carcinomas. This is above the percentage reported by other investigators who have used feeding experiments. Bielschowsky (*6*) obtained 64 percent breast cancers in 36 rats; Cox and associates (*7*) obtained 31 percent; Harris (*9*)

induced breast cancers in 62 percent of 18 female rats. Eighty-two percent (14 of 17) of female rats fed *N*-2-fluorenylacetamide developed mammary cancer during experiments conducted by Miller *et al.* (*10*). From the feeding experiments cited in this report there is evidence that *N*-2-fluorenylacetamide is absorbed from the gastrointestinal tract and carried via the circulation to the various responsive tissues and organs. In our experiment, absorption of the carcinogen must have been from the large bowel, although we were unable to determine the specific site. From the gross and histologic examination of the large bowel we found no evidence to suggest that absorption was not through the intact mucosa. No ulcers or breaks in the mucosa were observed. Unexplainable to us is the high selectivity of the carcinogen for the breast tissue, after reaching the circulation. Few other tumors were encountered. In comparison, Miller *et al.* (*10*) noted 2 liver tumors, 11 ear-duct tumors, and 2 small-bowel tumors in 19 female rats fed *N*-2-fluorenylacetamide.

Summary

1) Seventy-three percent of 67 female rats of the Sprague-Dawley strain developed breast tumors after weekly intracecal injections of *N*-2-fluorenylacetamide.

2) There was an indirect correlation between the incidence of breast tumors and the age at onset of injections.

3) No tumors of the large bowel and only a few tumors of other organs or tissues were encountered.

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PLATE

PLATE 35

FIGURE 1.—A cecal pouch (*arrow*) in the lower mid-line of the abdomen. The other masses in the milk line are breast tumors.

414469—57——16

The Responsibilities of the Medical Profession in the Use of X Rays and Other Ionizing Radiation

Statement by the United Nations Scientific Committee on the Effects of Atomic Radiation

Introduction

The United Nations General Assembly, being aware of the problems in public health that are created by the developments of atomic energy, established a Scientific Committee on the Effects of Atomic Radiation. This Committee has considered that one of its most urgent tasks was to collect as much information as possible on the amount of radiation to which man is exposed today, and on the effects of this radiation. Since it has become evident that radiation due to diagnostic radiology and to radiotherapy constitutes a substantial proportion of the total radiation received by the human race, the Committee considers it desirable to draw attention to information that has been obtained on this subject.

Modern medicine has contributed to the control of many diseases and has substantially prolonged the span of human life. These results have depended in part on the use of radiation in the detection, diagnosis and treatment of disease. There are, however, few examples of scientific progress that are not attended by some disadvantages, however slight. It is desirable therefore to review objectively the possible present or future consequences of increased irradiation of populations which result from these medical applications of radiation.

General Survey of the Irradiation of Human Beings

Man has always been exposed to some irradiation from natural sources. To this has now been added, as a result of modern discoveries and the applications of ionizing radiation and radioactivity, certain forms of artificial irradiation.

Natural irradiation is due to:

(1) cosmic radiation;

(2) "background" gamma radiation from radioactive substances present locally in the earth, rock or building materials, and from disintegration products of radon in air;

(3) radiations emitted from natural radioelements such as potassium 40, radium, radon and carbon 14, which are incorporated in the body.

The amount of this natural radiation varies with locality, but has

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been estimated as usually delivering between 70 and 170 mrems per year to the gonads. Of this total, the major contributions are about 45 percent from local gamma radiations, 30 percent from cosmic rays and 20 percent from body potassium 40.[1]

Artificial irradiation is derived from:

(1) the contamination of the environment, the atmosphere, or water by radioactive waste from atomic industries or from users of radioelements;

(2) the radioactive fallout, at greater or lesser distances from the source, or radioactivity resulting from the explosion of nuclear devices;

(3) the occupational exposure of certain groups of workers: medical practitioners, radiologists, dentists, nurses, atomic energy workers, uranium or thorium miners, and the industrial or scientific users of radiation generators or radioactive isotopes;

(4) the medical use of X rays, other ionizing radiations and radioelements in the detection, diagnosis, investigation and treatment of human diseases;

(5) the use of certain devices which emit radiation, such as television receivers, watches with luminous dials, and the X-ray generators used for the purpose of fitting shoes.

The amount of artificial radiation must vary considerably in different countries and we have inadequate information as to the over-all significance of these factors. In certain countries where estimates have been made, it appears that the greatest gonad irradiation of the population is due to diagnostic radiological procedures, the amount from this source about equaling that from all natural sources in certain instances. The total present contribution from occupational exposure, from the products of atomic industries, from radiotherapy and from the radiating devices mentioned above (paragraph 6, sub-paragraph 5), is likely to be very considerably smaller. That from radioactive fallout to the gonads appears at present to be in the region of 1 percent of the natural gonad irradiation in most areas.[2]

Both the magnitude and the significance of these various sources are under review by the Committee. Since medical irradiation accounts for a substantial if not the major proportion of all artificial exposure, it is important that its magnitude should be known accurately for different countries and circumstances. The possibility of making such an assessment depends upon the help of the medical profession, and particularly on the adequacy and availability of records kept by doctors, dentists and organizations responsible for the use of ionizing radiation.

Radiation Hazards

The medical use of radiation is clearly of the utmost value in the prevention, diagnosis, investigation and treatment of human disease, but the possible effects of this irradiation of individuals require examination.

[1] From reports sent by India, Sweden, the United Kingdom and the United States of America.

[2] According to the reports sent by the United Kingdom and the United States of America.

Generally speaking, the irradiation of living beings may produce radiobiological effects either on the irradiated individual himself or, through him, on his descendants; the former being termed somatic and the latter genetic effects. Somatic effects vary according to the different organs or tissues affected, and range from slight and reversible disturbances such as cutaneous erythema to the induction of leukemia or of other malignant diseases. The possible reversibility of the somatic effects of radiation received in small doses or at low dose rates encourages the belief that there are permissible doses of radiation which will not cause completely irreversible or significant somatic damage. The threshold for occasional somatic damage may, however, prove to be low. In the case of genetic effects, on the other hand, there may be no threshold. These effects increase with a frequency corresponding to the total amount of radiation received by the germinal tissues, and in the great majority of cases, are adverse.

Many other factors complicate the interpretation of radiobiological effects. The differences between whole and partial body irradiation, between a single exposure and continuous irradiation, or between the effects of different types of radiation are still imperfectly understood. Biological differences in the radiosensitivity of various tissues, or of the tissues of people of different age or sex, obviously influence the nature of radiation hazards. It is clear, however, that any irradiation of gonads, and any substantial irradiation of other tissues, involve a chance of significant damage which requires assessment.

General Recommendations Regarding the Medical and Occupational Irradiation of Human Beings

The radiological profession, through the International Commission on Radiological Protection,[3] has undertaken a valuable and responsible duty in defining maximum permissible limits of exposure for the main radiation hazards.

The establishment of these maximum permissible levels for those who are occupationally exposed to radiation depends on the view that there are doses which, in the light of our present knowledge, do not cause detectable somatic injury in the individual irradiated, and on the consideration that the number of individuals concerned is small enough for the genetic effects on the whole population to be negligible. For the gonads, or for irradiation of the whole body, the levels are such as to exclude doses greater than 0.3 rem in any week or 3.0 rem in any 13 weeks, or a sustained irradiation rate greater than 5 rem per year. These values imply that no total dose of over 50 rem will have been received by the gonads by the age of 30, or of over 200 rem by the whole body by the age of 60, in any occupationally exposed person.[3]

[3] See the report of the International Commission on Radiological Protection, published in the *British Journal of Radiology*—Supplement 6, of December 1954, in the *Journal Français d'electro-radiologie*—No. 10, of October 1955, etc.

As regards irradiation of the whole population, it is considered prudent to limit the average dose to germinal tissues from artificial sources to the order of magnitude of that received from all natural sources.

In considering the extent to which the population is irradiated for medical purposes, it is essentially the genetic hazard which is involved although it seems possible that in certain circumstances somatic injury may occur occasionally after low doses of radiation arise. Otherwise, the relevant dose is that indicating the mean gonad irradiation among the population as a whole up to the end of the average reproductive period.

The extent of such genetic irradiation from diagnostic procedures has been found to be equal to at least 100 percent of all natural radiation in two countries,[4] and that from a third equaled at least 22 percent of this figure.[5] Even before obtaining more exact values for these and other countries, it is clear that the exposure can be substantial in countries with extensive medical facilities, and that it is essential to consider any ways in which this exposure could be reduced without detriment to the existing or developing value of medical radiology.

The Committee is therefore anxious to obtain the help of radiologists in suggesting through appropriate governmental channels any methods by which this total exposure could be reduced and in estimating the amount of reduction that might be expected from any such methods. In particular it would be valuable to know how much the radiation to the gonads could be reduced:

(1) by improved design or shielding of equipment;

(2) by fuller training of any individuals using radiographic or fluoroscopic equipment;

(3) by any local shielding of the gonads that is practicable, especially during abdominal or pelvic examination;

(4) by the use of techniques involving radiography rather than fluoroscopy when full information can be obtained by this means;

(5) by improvement of administrative arrangements designed to obviate unnecessary repetition of identical examinations of the same subject;

(6) by a general study of certain medical conditions, such as that of peptic ulcers, to identify the circumstances in which the establishment of a radiological diagnosis has or has not a definite influence upon the treatment or prognosis given.

Summary

1) The Scientific Committee on the Effects of Atomic Radiation established by the United Nations General Assembly accepts the view that the irradiation of human beings, and especially of their germinal tissue, has certain undesirable effects.

2) Information received so far indicates that, in certain countries (Sweden, United Kingdom, United States of America), by far the most

[4] Sweden, United States of America.
[5] United Kingdom.

important artificial source of such irradiation is the use of radiological methods of diagnosis and that this may be equal in importance to radiation from all natural sources. It is possible that such radiation may be having a significant genetic effect on the population as a whole.

3) The Committee is fully aware of the importance and value of the medical use of radiations but wishes to draw the attention of the medical profession to these facts and to the need for a more accurate estimate of the amount of exposure from this source. The help of the medical profession would be most valuable to make it possible to obtain fuller information on this subject.

4) The Committee would be particularly grateful for information through appropriate governmental channels on ways in which the medical irradiation of the population can be reduced without diminishing the true value of radiology in diagnosis or treatment.

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